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(71) Applicant *(for all designated States except US)*: STICHTING DIENST LANDBOUWKUNDIG ONDERZOEK [NL/NL]; Bornsesteeg 53, NL-6708 PD Wageningen (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MOORMANN, Robertus, Jacobus, Maria [NL/NL]; Telgang 12, NL-8252 EH Dronten (NL). BOUMA, Annemarie [NL/NL]; Singel 6 II, NL-1013 GA Amsterdam (NL). VAN RIJN, Petrus, Antonius [NL/NL]; Gaastmeerstraat 2, NL-8226 HV Lelystad (NL). DE SMIT, Abraham, Johannes [NL/NL]; Patrijs 10, NL-8251 MB Dronten (NL).

(74) Agent: OTTEVANGERS, S., U.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).

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(54) Title: PESTIVIRUS VACCINATION

(57) Abstract

The invention relates to the field of eradication or control of viral diseases of animals, more specifically to vaccines used in the eradication or control of pestivirus infections in pigs or ruminants. The invention provides a recombinant nucleic acid derived from a genotype of a pestivirus, and chimeric virus and vaccine derived thereof, wherein a first fragment or part thereof encoding at least one immunodominant part at least partly responsible for providing protection against a wild-type pestivirus infection of a viral protein has been modified to render said immunodominant part incapable of eliciting antibodies specific for said genotype.

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Title: Pestivirus vaccination

The invention relates to the field of eradication or control of viral diseases of animals, more specifically to vaccines used in the eradication or control of pestivirus infections in pigs or ruminants.

Vaccination can serve two purposes: 1) to protect the vaccinated individual against signs of the disease in case of contact with the agent (individual immunity), and 2) to reduce each individual's chance of becoming infected when it is a part of a population of vaccinated individuals (herd immunity). The development of marker vaccines for veterinary use has led to new concepts for control and eradication of economically important viral diseases of livestock. The use of a marker vaccine allows serological discrimination between vaccinated and field-virus infected animals, and thereby a controlled elimination of the virus.

For instance, in most member states of the EU vaccination against Aujeszky's disease (AD) is allowed with gE-negative (deleted) vaccines only. Animals infected with an AD field virus do develop antibodies against gE. An ELISA test which detects these antibodies is used for the detection of infected animals, which subsequently may be removed from a herd, and for monitoring the status of wild-type Aujeszky's disease virus (ADV) infections in a herd during or after vaccination campaigns. Eventually, the aim is to reach a field or wild-type virus free status of a herd. Vaccination can then be discontinued and a serological surveillance program to guard this status should come into force.

However, said marker technology which is used in the case of ADV, a large DNA virus, simply by deleting fragments from the genome of said virus, can not be used with small RNA viruses, such as *Pestiviruses*, whereby it is impossible to delete fragments from the small RNA genome without disrupting its viability.

The genus *Pestivirus* of the family *Flaviviridae* conventionally consists of classical swine fever virus (CSFV), Border disease virus (BDV), and bovine viral diarrhoea virus (BVDV). Genomes of several BDV and CSFV strains have been sequenced, individual pestiviral proteins have been expressed and infectious copy RNA viruses have been generated (Renard et al., 1987 EP application 0208672; Collett et al., 1988, Virology 165, 191-199; Deng and Brock, 1992, Virology 1991, 865-679; Meyers et al., 1989, Virology 171, 555-567; Moormann et al., 1990, Virology 177, 184-188; Meyers et al., 1989, EP 89104921; Moormann and Wensvoort, 1989, PCT/NL90/00092; Moormann and Van Rijn; 1994, PCT/NL95/00214; Ridpath et al., 1997, Virus Res. 50: 237-243; Becker et al., 1998, J. Virol. 72:5165-5173).

The pestivirus genome is a positive-stranded RNA molecule of about 12.5 kilobases containing one large open reading frame. The open reading frame is translated into a hypothetical polyprotein of approximately 4,000 amino acids, which is processed by virus- and cell-encoded proteases. The open reading frame is flanked by two highly conserved small nontranslated regions, which are probably involved in the replication of the genome. The 5'-noncoding region also plays a role in initiation of translation.

The polyprotein which is co- and posttranslationally processed by cellular and viral proteases contains all the viral structural and nonstructural proteins (for review see C.M. Rice: In Fields Virology, Third Edition, 1996) *Flaviviridae*: The Viruses and their Replication: Chapter 30: pp. 931-959. The viral structural proteins, among which the envelope proteins E^{rns}, E1 and E2, are located in the N-terminal part of the polyprotein. The nonstructural proteins among, which the serine protease NS3 and RNA replicase complex NS5A and NS5B, are located in the C-terminal part of the polyprotein.

To date, BDV and BVDV have been isolated from different species (ruminants and pigs), whereas CSFV seems to be

restricted to swine. Pestiviruses are structurally and antigenically closely related.

Envelope glycoprotein E2 is the most immunodominant and most variable protein of pestiviruses. We cloned E2 genes of many different pestivirus strains, including those from a deer and a giraffe. The E2 genes were transiently expressed, characterised with monoclonal antibodies, sequenced and compared, P.A. van Rijn et al., 1997, Virology, 237: 337-348. Based on these data, we can delineate six major genotype groups within the pestivirus genus which each correspond to genotype specific E2 protein. Within groups, the antibodies against E2 strongly cross-react, in ELISA as well as in neutralisation tests, in between genotype groups, E2 proteins are in general antigenically unrelated and E2-sera or E2-specific antibodies against one genotype are not or only little reactive in inter-genotype ELISA or neutralisations tests with any one of the other genotype E2 proteins.

Four genotype groups correspond to the defined traditionally known genotypes CSFV, BVDV and BDV, whereas the two other groups are relatively new genotypes within the pestivirus genus.

One group (genotype CSFV) comprises CSFV strains isolated from swine. A second group (genotype BDV) comprises BDV strains Moredun, L83 and X818, which have been isolated from sheep, and strain F from swine. A third group (genotype BVDV II) comprises strain BD78 from sheep, strain 5250 from swine and strain 178003 from cattle. On the basis of E2, these viruses are very similar to BVDV strains associated with acute severe outbreaks of bovine viral diarrhoea, and comprise the so called type II BVDV. The fourth group (genotype BVDV type I) comprises BVDV strains predominantly originating from cattle. This BVDV-group can be divided into two subtypes or subgroups BVDV-1a and 1b: BVDV-1a comprises viruses from the USA, like NADL and Oregon, and some others, like 150022 and 1138 from Europe. Subgroup BVDV-1b comprises strain Osloss and several Dutch isolates. The fifth (genotype

Deer) and sixth (genotype Giraffe) "group" could be proposed as two new genotypes and contain strains Deer and Giraffe, respectively.

In general, antibodies raised against one genotype pestivirus are only of little protective value when tested with other genotype-specific wild-type pestivirus infections, no or only little cross-protection between genotypes exists.

Animals infected with a pestivirus develop detectable antibodies against E^{rns}, E2 and NS3. Antibodies directed against E2 are strongly virus neutralising and are generally thought to be the most protective within the genotype tested. Considering the central role antibodies directed against E2 play in protection, most focus has been given at developing marker vaccines comprising E2 or (recombinant) E2 subunit vaccines, all capable of generating protective antibody levels directed against E2 to be used against pestivirus infections. Antibodies directed against E^{rns} in general are genotype specific, have in general a low virus neutralising capacity and are considered having limited but additive protective capacity; those directed against NS3 have no neutralising capacity at all and are thought not to be protective, despite the fact that these are often broadly reactive within and between the several genotype groups.

CSFV is a notifiable disease for which in general the veterinary authorities are responsible for its eradication. Eradication generally occurs by a stamping-out policy, whereby all animals in an infected outbreak area are killed and destroyed, in the hope of thereby destroying the virus and preventing further transmission to other populations.

Authorities go to great length and costs in stamping-out, during the (latest) CSFV-epizootic in the Netherlands in 1997-1998, a estimated total of 9.000.000 pigs (on a total pig population of approximately 15.000.000) were killed and destroyed in relation to the outbreak. Such costs would markedly be reduced when vaccination was used to protect

populations at risk, however, current available vaccines are in general not deemed fit for the purpose.

Several arguments against CSFV vaccination using currently available vaccines are used. For example, it is reasoned that vaccination clouds the issues. It complicates the diagnosis of the disease, particularly by serological testing when post-vaccination antibodies cannot be distinguished from post-infection antibodies and thereby masks occurrence of the disease. Furthermore, it is reasoned that vaccination does not protect every animal, some animals may harbour the causal agent, becoming latent carriers and perpetuating the disease. Also, vaccination hampers the free trade in animals, particularly for export purposes when vaccinated animals cannot be identified, restricting income from export from vaccinated regions.

When applied, CSFV vaccination would generally be performed during a mass campaign in an area where an outbreak of CSFV has occurred. This calls for rapid vaccination of large numbers of animals in a relatively short period. In such a mass campaign it is of imminent importance that an adequate protection at the herd level (the number of pigs that are protected against the wild type virus infection) is achieved rapidly. Using whole killed CSFV vaccines has been deserted long ago due to their lack of antigenic mass and thus protection. E2 subunit vaccines in general are difficult to produce in the large quantities needed for mass campaigns. Waiting for several weeks after a first vaccination for a second vaccination, as is required for at least one subunit CSFV vaccine in order to achieve protection, greatly hampers and delays the control of the disease and does not provide the required rapid herd immunity. Using a non-marker vaccine, such as the C-strain virus, does not provide the required serological discrimination whereby wild-type infected animals or populations can be identified and culled. Recombinant ADV-CSFV-E2 marker vaccine, in theory a vaccine carrying many desired characteristics to try to achieve successful

vaccination (it is live, its is a marker, it protects) also generates antibodies directed against ADV, which makes it impossible to use where pigs have maternal or otherwise (via vaccination or wild-type infection) derived antibodies directed against ADV. Furthermore, its use in CSFV eradication would frustrate the serological detection and eradication of ADV-infected pigs, and thus in the end be counterproductive as a whole to the pig industry.

In short, current available vaccines against CSFV (reviewed in Moormann et al., 1996, Proc. 14th Intern. Pig. Vet. Society Congress, pp. 25-29, Bologna, Italy), such as the classical vaccine C-strain virus (non-marker), the recombinant ADV-CSFV-E2 marker vaccine, and a E2 sub-unit vaccine are at present all considered to be insufficient by the authorities to reach one or more of above defined goals relating to immunity, therefore a need for a better CSFV vaccine exists.

The same goes for the other pestiviruses such as BVDV or BDV, despite the fact that these do not cause notifiable disease. With BVDV and BDV, which cross-infect between ruminant species and even can cause infections in pigs, live vaccines in general are not sufficiently broadly immuno-reactive, and cannot prevent or (to the contrary) even cause vertical transmission from infected cow or sheep to its foetus, thereby enhancing further transmission of the virus. Serological discrimination in case of BVDV is further hampered by the existence of the various serotypes (BVDV1a, BVDV1b, or BVDV2). Whole killed vaccines in general do not have enough antigenic mass to protect at all, or only for a short period, making repeated vaccinations necessary and (experimental) subunit BVDV vaccines suffer from the antigenically narrow spectrum they can cover.

The invention provides a recombinant nucleic acid derived from a genotype of a pestivirus wherein a first

fragment or part thereof encoding at least one immunodominant part at least partly responsible for providing protection against a wild-type pestivirus infection of a viral protein has been modified to render said immunodominant part incapable of eliciting antibodies specific for said genotype. Surprisingly, by rendering said immunodominant part less immunogenic and thus less protective, still a protective vaccine can be provided, as demonstrated below, that, however, allows for the serological discrimination needed in control and/or eradication programmes whereby vaccinated animals or (partly) vaccinated populations of animals or tested (and subsequently culled) for the presence of antibodies against the wild-type virus causing the disease under control.

It is than essential that the RNA genome in some way remains its ability to replicate, therefore, the nucleic acid modification, as provided by the invention, eventually allows for generating replicating virus (chimeric virus) comprising most of the original genome, supplemented with a modified part. Such a modification as provided by the invention lives up to the structural requirements of both the encoding RNA and of the encoded proteins, thereby allowing replication of the virus and translation of the genome in structurally relevant proteins with which a viable virus particle can be generated. Such modifications can for example comprise deleting or introducing a codon encoding cysteine, thereby allowing for disrupted or enhanced disulfide bridge formation in between viral proteins, thereby effecting dimerisation or multimerisation. Also, the invention provides deleting small fragments, often only single codons, or at least at several positions, to render the protein with the desired antigenic characteristics.

In a preferred embodiment the invention provides a recombinant nucleic acid derived from a genotype of a pestivirus wherein a first fragment encoding at least one immunodominant part, at least partly responsible for

providing protection against a wild-type pestivirus infection, of a pestiviral protein has been replaced by a second fragment encoding an antigenically distantly related or unrelated part of a pestiviral protein from another genotype. Herewith the structural requirements are particularly well met. Constructing or producing a recombinant nucleic acid as provided by the invention in essence comprises two aspects. A first aspect, relating to the selection of a part of a pestiviral protein selected for replacement, a second aspect relating to the part of pestiviral protein of the desired wild-type virus by which serological discrimination is enabled. These two parts are the same; often, an immunodominant protein elicits antibodies by which an infection can be detected, but it is the line of expectations that the same antibodies are at least partly protective, especially when these are genotype specific. For serological discrimination of pestivirus genotypes it does not suffice providing protection with a vaccine comprising the protective proteins E2 or E^{rns} but not the non-protective NS3 protein, and detecting infections with tests based on NS3. NS3 is not genotype specific, at least it does not elicit genotype specific antibodies to allow discrimination between genotypes. Such diagnostic tests can thus hardly be used in the aftermath of vaccination campaigns against for example CSFV in pigs. Circulating NS3-BDV or NS3-BVDV antibodies will cause a plethora of false-positive results, leading to suspicions of CSFV infections when in fact there aren't any in the pig population tested.

In constructing a vaccine, one has to take into account what (type of) serological test is preferred once the vaccine is employed in the field. It should be genotype specific, which blocks using diagnostic tests based on NS3. However, selecting E2 or E^{rns} as diagnostic antigens hampers developing a vaccine which uses the protective properties of these proteins. The invention surprisingly provides vaccines in which specific immunodominant parts, in general thought

responsible and necessary for generating protection, have been modified or replaced with antigenically unrelated or only distantly related (for that genotype) parts, thus allowing serological discrimination, without seriously hampering protective properties.

Contrary to what seems common sense in producing a vaccine, especially in the field of pestiviruses where so much focus has been given to the presence of protective E2 protein (of which, in a preferred embodiment of the invention, a protective part is at least partly replaced by an antigenically unrelated part of E2 of another genotype pestivirus), the invention provides pestiviral recombinant nucleic acid which has been deprived from at least part of its (genotype specific) protective capacities to allow for serological detection of wild-type infections with said genotype.

In a preferred embodiment of the invention a nucleic acid is provided wherein said viral protein is an E2 protein, for example wherein said first nucleic acid fragment comprises a nucleic acid encoding E2 protein from about amino acids 690 to 865 or 692 to 877 or 690 to 1008 or related positions in said pestivirus polyprotein sequence. For simplicities sake the numbering of the C strain sequence is used here for all pestivirus sequences. In fact in other pestivirus sequences the numbering of the E^{RNS} and the E2 proteins in the polyprotein may differ slightly due to length differences in the polyprotein sequences of pestivirus strains. Based on homology, the N and C termini of the E2 or E^{RNS} sequence of any pestivirus strain can, however, easily be determined.

The invention further provides a nucleic acid wherein said protein from another genotype pestivirus is an E2 protein, for example wherein said second fragment comprises a nucleic acid fragment encoding a part of the E2 protein of another pestivirus running from about amino acids 690 to 865

or 692 to 877 or 690 to 1008 in the polyprotein sequence of said other pestivirus.

In yet another embodiment, the invention provides a nucleic acid according wherein said viral protein is an E^{rns} protein, for example wherein said first fragment comprises a nucleic acid fragment encoding the E^{RNS} protein of a pestivirus running from about amino acids 268 to 494 in said pestivirus polyprotein sequence.

1. In addition, a nucleic acid according to the invention is provided wherein said protein from another genotype pestivirus is an E^{rns} protein, for example wherein said second fragment comprises a nucleic acid fragment encoding the E^{RNS} protein of another pestivirus running from about amino acids 268 to 494 in said other pestivirus polyprotein sequence.

The invention also provides a protein encoded by a nucleic acid according to the invention which is used as antigen in a serological test which discriminates animals infected with a wild type or field strain pestivirus from uninfected animals or animals vaccinated with an accompanying (live virus) pestivirus marker vaccine as provided by the invention

As indicated above the marker vaccine concept relying on serological discrimination between infected and uninfected or vaccinated animals involves the antigenic pestiviral proteins E^{RNS}, E2 and NS3. A discriminating test detecting antibodies against NS3 is unsuitable in combination with a pestivirus marker vaccine, based on the concept of exchanging antigenically unrelated or distantly related (parts of) proteins between pestivirus genotypes since the protein is highly conserved, and antibodies against NS3 are highly cross-reactive among pestiviruses.

However, the E^{RNS} and E2 proteins or parts thereof are suitable for this approach to pestiviral marker vaccine development.

In example 1 of this invention variants of the CSFV C strain and several preferred approaches to the "exchange

concept" are described. Now that this "exchange concept" approach has been described for generating a CSFV C strain marker vaccine, similar approaches can of course now be followed for any other pestivirus vaccine strain, irrespective of how this strain was generated before it was turned into a marker vaccine strain.

As far as the E^{RNS} protein is concerned a first nucleic acid fragment encoding the part of the polyprotein sequence of the C strain running from amino acid 268 to 494, was replaced by a second nucleic acid fragment encoding the E^{RNS} protein, amino acid 268 to 494 in the polyprotein sequence, of BVDV type II strain 5250 (Fig. 2D). The resulting chimeric C strain virus was designated Flc11. Since the complete sequence of strain 5250 was not available the N and C terminus of the E^{RNS} protein of this strain was determined on the basis of homology between pestivirus sequences (Fig.1A).

It should be stressed that the antigenic part of E^{RNS}, essential to this approach, may be N or C terminally or both, shorter or longer than the sequence exchanged in Flc11. Once established , these parts are as suitable for exchange as the complete E^{RNS} protein, and therefor are also provided. An identical replacement of the E^{RNS} protein of the C strain by the E^{RNS} protein of strain 5250 as described for Flc11 was performed in strain Flc7 (compare fig. 2B). In Flc7 a second first nucleic acid fragment encoding the antigenic N-terminal part of the E2 protein of the C strain running from amino acid 692 to 877 in the polyprotein sequence of the C strain, was replaced by a second nucleic acid fragment encoding the antigenic N-terminal part of the E2 protein of CSFV strain Brescia running from amino acid 692 to 877 in the polyprotein sequence of this strain. As is indicated in figure 1B these fragments essentially comprise an antigenic N-terminal part of the E2 protein of pestiviruses running from amino acid 690 to 865 (van Rijn et al., 1997. Virology 237:337-348). In chimeric virus Flc9 a first nucleic acid fragment encoding this part of the E2 protein of the C strain was replaced by a

second nucleic acid fragment encoding the identical part (amino acids 690 to 865, compare fig.1B) of E2 of BVDV type II strain 5250 (Fig. 2C). Another chimeric virus suitable as CSF live virus marker vaccine is virus strain Flc6 in which a first nucleic acid fragment encoding the nearly complete E2 protein running from amino acid 690 to 1008 in the polyprotein sequence of the C strain was replaced by a second nucleic acid fragment encoding E2 of BVDV strain NADL running from amino acid 693 to 1012 in the polyprotein sequence of this strain (Collett et al. 1988. Virology 165:191-199). In conclusion as far as the exchange of E2 protein encoding nucleic acid fragments between pestivirus genotypes for development of pestiviral marker vaccines is concerned, two preferred embodiments have been described in this invention:
1) exchange of the region essentially comprising sequences encoding the antigenic N-terminal half of the protein i.e. amino acid 690 to 877 (numbering of C strain sequence). As shown in this invention, a number of amino acids extra or less at the N (690) and/or C (877) terminus still results in suitable pestivirus marker vaccines.
2) exchange of the region encoding a (nearly) complete E2 protein sequence i.e. amino acid 690 to 1008 (numbering of C strain sequence).

The invention provides a chimeric pestivirus wherein nucleic acid encoding at least a part of at least one pestiviral protein has been replaced by nucleic acid encoding a structurally related part of at least one other pestiviral protein allowing serological discrimination of an animal vaccinated with said vaccine from an animal infected with wild-type pestivirus. A vaccine as provided by the invention preferably comprises a live chimeric pestivirus (and thus preferably is a live vaccine). For a (killed) vaccine comprising a killed chimeric pestivirus substantially more antigenic mass is needed to provide the desired effects, although in principle the effects, relating to protection as well as to serological discrimination, once having been

achieved by a killed vaccine, have similar advantages as with a live vaccine.

In a much preferred embodiment, the invention provides a vaccine according to the invention capable of providing sufficient herd immunity to a vaccinated population to reduce the reproduction ratio (R_0) of said wild-type pestivirus to at least below 1, thereby allowing sufficient reducton of viral transmission in vaccinated populations to eradicate or control the infection in the population.

In a preferred embodiment nucleic acid or a chimeric pestivirus according to the invention is derived from a pestivirus vaccine strain, such as C strain virus the Japanese GPE- strain virus (Sasahara et al. 1969. Nat. Inst. Of Animal Health Quarterley 9:83-91) or the French Thiverval strain (Aynaud. 1976. EEC Report, EUR 5486, C.E.C. Luxembourg, p.93-96) derived from a classical swine fever virus. Although the invention has been described for a live CSF vaccine strain attenuated by classical techniques, it can equally well be applied to other pestiviral vaccine strains that have been attenuated or generated by e.g. recombinant DNA techniques.

The invention provides a recombinant nucleic acid wherein surprisingly at least a part of a pestiviral protein introduced to allow broad serological discrimination is stably maintained, thus providing the so desired marker characteristics. For example, our experimental results are that Flc6, Flc7, Flc9 and Flc11 stably maintain the heterologous E2 and/or E^{RNS} genes in their otherwise CSFV C-strain genetic make-up. This despite the fact that Flc6 and Flc9 needed adaptation to SK6.T7 cells and grow to lower titres than Flc7, Flc11 and Flc2.

In a preferred embodiment of the invention said chimeric pestivirus comprises Flc7, deposited as number I - 2059, at 31 July 1998, at the CNCM, Paris, France.
Strain Flc7 is especially suitable for use as a CSF marker vaccine for emergency vaccination to control outbreaks of CSF

in pig areas where there is normally no vaccination against CSF applied and where rapid immunity is required. However, Flc7 is equally suitable as vaccine for prophylactic vaccination in areas where CSF is endemic. Optimal benefit of Flc7 is achieved if the vaccine is used in combination with the serological test which specifically detects antibodies against E^{RNS} of CSFV, and which discriminates the animals infected with the wild type or field CSF virus from the uninfected animal or the animal vaccinated with Flc7. In this way Flc7 allows the specific detection of the CSFV infected animal or herd in the vaccinated population, which animal or herd can than be specifically removed.

In a preferred embodiment of the invention said chimeric pestivirus comprises Flc9, deposited as number I - 2060, at 31 July 1998, at the CNCM, Paris, France.
Strain Flc9 is especially suitable for use as a CSF marker vaccine for emergency vaccination to control outbreaks of CSF in pig areas where there is normally no vaccination against CSF applied and where rapid immunity is required. However, Flc9 is equally suitable as vaccine for prophylactic vaccination in areas where CSF is endemic. Optimal benefit of Flc9 is achieved if the vaccine is used in combination with the serological test which specifically detects antibodies against E2 of CSFV, and which discriminates the animals infected with the wild type or field CSF virus from the uninfected animal or the animal vaccinated with Flc9. In this way Flc9 allows the specific detection of the CSFV infected animal or herd in the vaccinated population, which animal or herd can than be specifically removed.

In a preferred embodiment of the invention said chimeric pestivirus comprises Flc11, deposited as number I - 2061, at 31 July 1998, at the CNCM, Paris, France. Strain Flc11 is especially suitable for use as a CSF marker vaccine for emergency vaccination to control outbreaks of CSF in pig areas where there is normally no vaccination against CSF applied and where rapid immunity is required. However, Flc11

is equally suitable as vaccine for prophylactic vaccination in areas where CSF is endemic. Optimal benefit of Flc11 is achieved if the vaccine is used in combination with the serological test which specifically detects antibodies against E^{RNS} of CSFV, and which discriminates the animals infected with the wild type or field CSF virus from the uninfected animal or the animal vaccinated with Flc11. In this way Flc11 allows the specific detection of the CSFV infected animal or herd in the vaccinated population, which animal or herd can than be specifically removed.

In a preferred embodiment of the invention said chimeric pestivirus comprises Flc6, deposited as number I - 2058, at 31 July 1998, at the CNCM, Paris, France. Strain Flc6 is especially suitable for use as a CSF marker vaccine for emergency vaccination to control outbreaks of CSF in medium pig areas where there is normally no vaccination against CSF applied and where rapid immunity is required. However, Flc6 is equally suitable as vaccine for prophylactic vaccination in areas where CSF is endemic. Optimal benefit of Flc6 is achieved if the vaccine is used in combination with the serological test which specifically detects antibodies against E2 of CSFV, and which discriminates the animals infected with the wild type or field CSF virus from the uninfected animal or the animal vaccinated with Flc6. In this way Flc6 allows the specific detection of the CSFV infected animal or herd in the vaccinated population, which animal or herd can than be specifically removed.

The invention furthermore provides a method for controlling and/or eradicating a wild-type pestivirus infection comprising vaccinating at least a part of an animal population with a vaccine according to the invention and further comprising testing at least a part of said population for the presence of antibodies specific for said wild-type virus.

Animals infected with an field virus develop antibodies against an immunodominant part of that virus. An ELISA or

neutralisation test (differentiating tests) as provided in the experimental part of this description which detects these antibodies is used for the detection of infected animals, which subsequently are removed from a population. The use of a pestivirus marker vaccine as provided by the invention wherein said immunodominant part has been modified or replaced allows serological discrimination between vaccinated and field-virus infected animals, and thereby a controlled elimination of the virus. These differentiating tests are also used for monitoring the status of wild-type pestivirus infections and transmission in a population during or after vaccination campaigns. Using a vaccine as provided by the invention capable of providing sufficient herd immunity to a vaccinated population to reduce said wild-type virus transmission to a reproduction ratio of < 1 greatly facilitates control and/or eradication. Eventually, the aim is to reach a field or wild-type virus free status of a herd. Vaccination can then be discontinued and a serological surveillance program to guard this status should come into force.

When an outbreak of CSFV is notified, eradication of the virus usually occurs by a stamping out policy. The availability of a CSFV marker vaccine now allows vaccination with such a vaccine as an additional tool to control the CSFV outbreak. A possible way to proceed is that all pigs of the infected herd will be destroyed and all pigs on farms in a certain zone around the infected herd are vaccinated with a marker vaccine as provided by the invention. The vaccine preferably should be administered within a short time (e.g. 2 weeks). The vaccination can possibly be followed by a follow-up vaccination, three to four weeks after the first vaccination.

After vaccination, a surveillance period will be started. During this period, which might take at least 2 months, all herds in the zone will be screened regularly for clinical symptoms of CSF, and serum samples will be

collected. These serum samples will be tested in the differentiating tests for antibodies directed against wild-type CSFV. Herds with pigs with antibodies directed against wild-type CSFV will be destroyed. Herds with pigs without antibodies directed against wild-type CSFV during a prescribed period should be declared negative for CSFV. The zone should be declared free of CSFV when during a prescribed period no antibodies against wild-type CSFV are detected on all herds in the surveillance zone. After the lift of movement restrictions, the CSFV-negative but vaccinated pigs can be traded and slaughtered. Policy makers, however, will further decide about the implementation of the vaccine and about the measures taken after vaccination, for example depending on regional or political preferences.

Likewise control and/or eradication measures are provided for BDV and BVDV. The invention is further explained in the experimental part of the description without limiting the invention.

Experimental part

Example 1

Construction and characterisation of chimeric pestiviruses

Cells and virus

Swine kidney cells (SK6-M, EP 0 351 901 B1) were grown in Eagle's basal medium containing 5% fetal bovine serum, glutamine (0.3 mg/ml), and the antibiotics penicillin (200 U/ml), streptomycin (0.2 mg/ml), and mycostatin (100 U/ml). Fetal bovine serum was tested for the absence of BVDV and BVDV antibodies as described previously (Moormann *et al.* 1990. Virology **177**:184-198). SK-6 cells expressing bacteriophage T7 RNA polymerase (SK6.T7; H.G.P.van Gennip *et al.* 1998. submitted) were grown in the same medium supplemented with 12.5 times diluted histidinol stock (125 mM histidinol in 100 mM HEPES).
Recombinant or chimeric C-strain viruses were grown and prepared as described earlier (Moormann *et al.* 1996. J. Virol.**70**:763-770) with slight modifications. The growth medium of SK6 cells was changed in supplemented Eagle's basal medium. Virus stocks were prepared by passaging the virus three times on SK6 cells. The virus stocks had titres of about $10^{4.0}$-$10^{6.5}$ $TCID_{50}$/ml. BVDV strain 5250 of porcine origin (Hooft van Iddekinge *et al.*, 1992. Vet. Microbiol. **30**:21-34; Wensvoort *et al.*, 1989. Vet. Microbiol. **20**, 291-306) was biologically cloned three times by end point dilution on bovine turbinate (BT) cells and was also grown on SK6 cells as described above.

Isolation of RNA from infected SK6 and BT cells

Intracellular RNA from cells infected with the recombinant C-strain viruses was isolated essentially as described (Moormann *et al.* 1990. Virology **177**:184-198).
Briefly, monolayers of SK6-M cells were infected with virus at a multiplicity of infection (m.o.i.) of 0.1. Cells were incubated for 1.5 h at 37°C, and fresh medium was added. After 4 days cells were washed twice with ice cold phosphate buffered saline (PBS), and lysed in ice-cold lysisbuffer (50 mM Tris-HCl pH 8.2, 0.14 M NaCl, 2 mM $MgCl_2$, 5 mM DTT, 0.5%[v/v] NP-40, 0.5%[w/v] Na-deoxycholate, and 10 mM vanadyl ribonucleoside complexes (New England Biolabs)). The lysates were centrifuged (4 °C, 5 min, 4000x *g*) and the supernatant was treated with proteinase K (250 µg/ml, final concentration) for 30 min at 37 °C, extracted twice with phenol, chloroform, and isoamylalcohol (49:49:2), and extracted once with chloroform and isoamylalcohol (24:1). RNA was stored in ethanol.
RNA from strain 5250 from BT cells was isolated as described previously (Hooft van Iddekinge *et al.*, 1992. Vet. Microbiol. **30**:21-34).

Cloning and sequencing of cDNA of the genes encoding E^{RNS} and E2 of BVDV strain 5250

The E2 gene of BVDV strain NADL was cloned and sequenced as described (van Rijn *et al.* 1997. Virology **237**:337-348), resulting in plasmid pPRKbvd10.
The antigenic region of the E2 gene of strain 5250 was cloned and sequenced as described (van Rijn *et al.* 1997. Virology **237**:337-348), resulting in plasmid pPRKbvd77.
RNA from BT cells infected with BVDV strain 5250 was isolated as described above and a fragment covering the E^{RNS} gene was

amplified from cDNA by reverse transcription-PCR (RT-PCR) essentially as described (van Rijn *et al.* 1994. J. Virol. **68**:3934-3942) using forward primer p343 (5'-CCCGGGATCCAAAAAGCCCTGTTGGC[A,T]TGGGC-3') and reverse primer p305 (5'-GGGGTGCAGTTGTTTGTATCCA-3'). The blunt PCR-products were cloned in pGEM4z-blue.
The nucleotide sequence of E^{RNS} of strain 5250 was determined by thermocycle sequencing (Perkin Elmer/Applied Biosystems Division) on two independently amplified and cloned fragments. Deduced amino acid sequences were compared with those of published BVDV I and II and BDV strains with the MacMolly-tetra multialignment program.

Construction of hybrid full-length DNA clones pPRKflc6, pPRKflc7, pPRKflc9 and pPRKflc11

pPRKflc6 is a full-length clone containing the C-strain genome in which the E2 gene between the *Spe*I and *Afl*II sites of pPRKflc2 (previously named pPRKflc-133, Moormann *et al.*, 1996. J. Virol. **70**:763-770), was replaced with the same region of BVDV strain NADL (Fig. 2A). The *Spe*I-*Sna*BI fragment of pPRKbvd10 (van Rijn *et al.* 1997. Virology **237**:337-348) was cloned in pPRc144, which is a derivative of pPRc129 (WO 95/35380) in which the *Spe*I site in the vector was mutated to make this site unique. The resulting plasmid, designated pPRKh1, was transfected into SK6.T7 cells and tested for expression of C-strain E^{RNS} and NADL E2 as described previously (van Rijn *et al.* 1993. J. Gen. Virol. **74**:2053-2060). C-strain E^{RNS} expression was detected with Mab C5 (Wensvoort, G. 1989. Epitopes on structural proteins of hog cholera (swine fever) virus. PhD thesis, State University of Utrecht), and NADL E2 expression was detected with Mab CT6 (Bolin *et al.* 1988. Arch. Virol. **99**, 117-123). Finally, the *Nco*I-*Xba*I fragment of pPRc132 (WO 95/35380) was inserted in *Nco*I-*Xba*I digested pPRKh1, resulting in the hybrid full-

length plasmid pPRKflc6 (Fig 2A).

pPRKflc7 is a derivative of pPRKflc3 (previously named pPRKflc-h6, Moormann *et al.*, 1996. J. Virol. **70**:763-770) in which the E^{RNS} gene of CSFV strain C between amino acids 267-495 (numbering of CSFV strain C; EMBL nr. Z46258) was replaced with the same region of BVDV strain 5250 (Fig. 2B). To this end, the E^{RNS} gene was PCR amplified. Plasmid pPRKbvd37 (unpublished), containing the E^{RNS} gene from BDVD strain 5250, was used as template DNA and amplified with forward primer p936 (5'-CCGAGAACATCACCCAGTGG-3') and reverse primer p937 (5'-CATGTGCTCCAAACCATGAT-3'). The PCR fragments were phosphorylated with T4 polynucleotide kinase (New England Biolabs) and isolated from an agarose gel. The isolated fragments were cloned in a calf intestinal phosphatase (New England Biolabs)-treated *Stu*I site of expression vector pPRKc5 (Hulst *et al.* 1998. J. Virol. **72**:151-157). Clones in which the 5250 E^{RNS} gene were inserted in the correct orientation were transfected into SK6 cells and tested for expression of 5250 E^{RNS} and C strain E2 as described above. E^{RNS} expression was detected with Mab WB433 (Paton *et al.* 1994. Arch. of Virol. **135**:241-252), and C-strain E2 expression was detected with Mab b3 (Wensvoort, G. 1989. J. Gen. Virol. **70**:2865-2876). From clone pPRKc13, which expressed both 5250 E^{RNS} and C-strain E2, a *Cla*I-*Ngo*MI fragment was inserted in the *Cla*I-*Ngo*MI digested pPRKflc3, resulting in full-length hybrid clone pPRKflc7 (Fig. 2B).

pPRKflc9 is a full-length clone containing the C-strain genome in which the antigenic region of E2 between the *Spe*I and *Bgl*II sites of pPRKflc3, was replaced with the same region of BVDV strain 5250 (Fig. 2C). To this end, the *Spe*I-*Sal*I fragment of pPRKbvd77 (van Rijn *et al.* 1997. Virology **237**:337-348) was cloned in expression vector pPRc83. This vector, a pEVhisd12 derivative (Peeters *et al.* 1992. J.

Virol. **66**:894-905), contains the structural genes of CSFV strain C (N^{pro}-C-E^{RNS}-E1-E2, amino acids 5 to 1063 of the sequence of CSFV strain C) (Moormann *et al.*, 1996. J. Virol.**70**:763-770). The resulting clone, designated pPRKh18, was transfected into SK6 cells and tested for expression of C-strain E^{RNS} and 5250 E2 as described above. Expression of C-strain E^{RNS} was detected with Mab C5 (see above), and expression of 5250-E2 was detected with Mab WB166 (Paton *et al.* 1992. Virology **190**:763-772). The *Cla*I-*Bgl*II fragment of pPRKh18 was inserted in *Cla*I-*Bgl*II digested pPRKflc3, resulting in the hybrid full-length plasmid pPRKflc9 (Fig. 2C).

pPRKflc11 is a full-length clone containing the C-strain genome in which the E^{RNS} gene of CSFV strain C between amino acids 267-495 (numbering of CSFV strain C; EMBL database nr Z46258) was replaced with the same region of BVDV strain 5250 (Fig. 2D). From clone pPRKc13, also mentioned above (Fig. 2B), a *Cla*I-*Ngo*MI fragment was inserted in the *Cla*I-*Ngo*MI digested plasmid pPRKflc2, resulting in the hybrid full-length clone pPRKflc11 (Fig. 2D).

Isolation of recombinant viruses Flc6, Flc7, Flc9 and Flc11

Plasmid DNA from pPRKflc6, pPRKflc7, pPRKflc9 and pPRKflc11 was purified on columns (Qiagen) and linearized with *Xba*I. The DNA was extracted with phenol-choroform, precipitated with ethanol, and dissolved in water. 25 ml of Optimem-I containing 1 mg Lipofectine (Gibco-BRL) was preincubated for 45 min at room temperature. Linearized DNA (200 ng) was diluted in 25 ml Optimem-I (Gibco-BRL) and mixed with the preincubated lipofectine mixture and incubated for 15 min at room temperature. SK6.T7 cells, grown in 2 cm^2 tissue culture plates, were washed once with Optimem-I. Fresh Optimem-I was

added (0.2 ml), followed by the DNA transfection mixture. After 5 h of incubation at 37°C, the transfection mixture was removed and the wells were supplied with fresh medium. The cells were incubated for 4 days at 37°C, after which the transfection supernatant was stored at -70°C. Cells were immunostained with Mabs C5 (for Flc6 and Flc9) and b3 (for Flc7 and Flc11). The medium collected from wells in which expression was observed was applied onto fresh SK6 or SK6.T7 cells to determine the presence of infectious virus. After four days, the monolayers were fixed and immunostained as described above.

Growth kinetics of Flc9 and Flc11

Growth kinetics of the viruses Flc9 and Flc11 were determined in SK6 cells. Subconfluent monolayers in T25 flasks (Costar) were infected at a multiplicity of infection of 0.1. Viruses were adsorbed for 1.5 h. Before cells were supplied with fresh medium, the first sample at time point zero was collected. Virus titres were determined separately from supernatants and cell lysates. To determine the virus titre in cells, cells were freeze/thawed twice at -70°C in 2 ml of growth medium and clarified for 10 min at 5000 x *g* at 4°C. Virus titres (log $TCID_{50}$ per millilitre) were determined at 0, 6, 24, 48, 72, 96, 122 and 144 h after infection.

Characterization of Flc6, Flc7, Flc9 and Flc11

The E^{RNS} genes of Flc7 and Flc11, and the E2 genes of Flc6 and Flc9 were sequenced. Cytoplasmic RNA of SK6 cells infected with the respective viruses was isolated as described above. DNA fragments covering the E^{RNS} genes of Flc7 and Flc11 and the E2 genes of Flc6 and Flc9 were amplified by RT-PCR and analysed on a 1.5% agarose gel in 1xTAE, and purified on Costar Spin-X columns. Sequences of the purified PCR fragments were determined by PCR cycle sequencing using the

dRhodamine dye terminator ready reaction cycle sequencing kit (PE/ABD) according to the manufacturers conditions with flanking and internal primers and analysed on a 310 ABI PRISM genetic analyser. In addition, viruses were characterised by an immunoperoxidase monolayer assay (Wensvoort, G. 1986. Vet. Microbiol. **12**, 101-108). For this test, SK6 cells were infected with viruses Flc2, Flc6, Flc7, Flc9, Flc11 and 5250. After incubation for 4 days at 37°C, monolayers were immunostained with Mabs specific for CSFV E2 (Mabs b6 and c2), BVDV E2 (Mabs WB166 and CT6), C-strain E^{RNS} (Mab C5), or BVDV E^{RNS} (Mab WB433).

Results

Cloning and sequencing of cDNA of the genes encoding E^{RNS} and E2 of BVDV strain 5250

The cloning and sequencing of the N-terminal part of E2 of BVDV strain 5250 has been described by Van Rijn *et al.*, 1997. (Virology 237:337-348). The cloning and sequencing of the E^{RNS} gene of this strain has not been published and is here described for the first time. Established and published amino acid sequences of 5250 E2 and E^{RNS} were aligned with those of BVDV type I strains NADL (Collett *et al.* 1988. Virology **165**:191-199) and Osloss (Renard *et al.* 1987. European patent application 86870095.6), BVDV type II strain 890 (Ridpath and Bolin. 1995. Virology **212**:39-46), CSFV strain C (Moormann *et al.* 1996. J. Virol. **70**:763-770), CSFV strain Brescia (Moormann *et al.* 1990. Virology **177**:184-198) and BDV strain BD31 (Sullivan *et al.*, 1997. Virus Res. **47**:19-29). This alignment clearly demonstrated that the 5250 E^{RNS} and E2 sequences are most homologous with those of BVDV strain 890 (Figs. 1A and 1B), and confirm the data of van Rijn *et al.* 1997 (ibid), who on basis of sequence comparison of E2, demonstrated that 5250 belongs to the BVDV type II category

of pestiviruses.

Construction and isolation of Flc6, Flc7, Flc9 and Flc11; production and growth kinetics of Flc9 and Flc11.

The construction of plasmids pPRKflc6, pPRKflc7, pPRKflc9 and pPRKflc11 which contain full-length DNA copies of the RNA genomes of CSFV C-strain viruses in which the E^{RNS} or E2 genes or both have been substituted, is shown in figures 2A-D. For recovery of recombinant viruses, full-length cDNA plasmids were transfected into SK6.T7 cells endogenously expressing the bacteriophage T7 RNA polymerase gene.
Infectious virus could be recovered from supernatants of SK6.T7 cells transfected with pPRKflc7 and pPRKflc11 but not from cells transfected with pPRKflc6 and pPRKflc9. However, because SK6.T7 cells transfected with pPRKflc6 and pPRKflc9 were positive after immunostaining, cells transfected in parallel were trypsinized, diluted 20-fold in Eagle's supplemented medium and grown for four days in two T25 flasks. The cells were passaged several times in the same way. After each passage, one T25 flask was freeze/thawed twice and the cell suspensions were clarified at 5000 x *g* for 10 minutes at 4°C. The virus titres of the clarified supernatants were determined by end point dilution. After three passages on SK6.T7 cells, virus titres increased to a maximum of 10^4 $TCID_{50}$/ml for Flc 6 and Flc9. In the same way virus stocks of about 10^5 $TCID_{50}$/ml of Flc7 and Flc11 were prepared.
Viruses Flc9 and Flc11 were further propagated on SK6 cells as described in the section "Cells and Virus". Virus stocks of Flc9 reached titres of 10^5-10^6 $TCID_{50}$/ml, whereas virus stocks of Flc11 reached titres of 10^6-10^7 $TCID_{50}$/ml.

Since Flc9 needed adaptation to SK6.T7 cells and seemed to grow to log10 lower virus stock titres, growth kinetics of

Flc9, Flc11 and the parent viruses 5250 and Flc2 were compared. As is shown in figure 3 the multistep growth curves of Flc9 and 5250 are very similar, and indicate a slower growth rate and about log10 lower virus titre plateau levels for these viruses, compared to Flc2 and Flc11. Also the growth curves of Flc2 and Flc11 are very similar, as are their virus titre plateau levels. This suggests that the growth rate of CSFV based chimeric pestiviruses in SK6 cells is more dependent on the presence in these constructs of CSFV E2 than on the presence of CSFV E^{RNS}. Probably this effect is exerted at the level of infection.

Characterization of Flc6, Flc7, Flc9 and Flc11.

Characterization of Flc6, Flc7, Flc9 and Flc11 at the molecular level was performed by sequencing their E2 (Flc6, Flc9) or E^{RNS} (Flc7, Flc11) genes. No sequence differences resulting in amino acid changes between the E2 gene of Flc9 and the E^{RNS} genes of Flc7 and Flc11, and the sequences of E2 and E^{RNS} of 5250, from which they were derived, were observed. The same result was found when the sequence of the E2 gene of Flc6 was compared with published NADL E2 sequences (Collett *et al.* 1988. Virology **165**:191-199; van Rijn *et al.* 1997. Virology **237**:337-348).

Furthermore, Flc6, Flc7, Flc9 and Flc11 were characterized by their specific reaction with a panel of CSFV and BVDV specific Mabs directed against E^{RNS} and E2 (Table 1). Flc9 and strain 5250, containing the E2 gene of BVDV type II strain 5250, specifically react with Wb166, a BVDV E2 specific Mab. Strains Flc9 and 5250 can be discriminated from NADL and Flc6, containing the E2 gene of BVDV type I strain NADL, by the specific reaction of another BVDV E2 specific Mab, CT6, with the latter two strains. As expected, strains Flc6, Flc7, Flc9, NADL, 5250, and Brescia do not react with Mab C2 which specifically reacts with strains Flc2 and Flc11, both

containing the E2 gene of CSFV strain C. Mab b6, which recognizes an epitope on E2 of CSFV strain Brescia specifically reacts with strains Flc7 and Brescia. Further analysis with pestivirus strain specific E^{RNS} Mabs resulted in a more refined antigenic typing of each of the above described (recombinant) pestivirus strains. The BVDV E^{RNS} specific Mab WB433 specifically reacts with strains Flc7, Flc11 and 5250, since these strains contain the BVDV specific E^{RNS} gene of strain 5250. In contrast, these strains do not react with the CSFV specific E^{RNS} Mab C5, which specifically reacts with strains Flc2, Flc6 and Flc9, containing the E^{RNS} gene of CSFV strain C.. Combining the data of the Mab typing on E^{RNS} and E2 resulted in unique reaction profiles with the Mabs for each of the strains in Table 1. In conclusion, these findings show that Flc6, Flc7, Flc9 and Flc11 stably maintain the heterologous E2 and/or E^{RNS} genes in their otherwise CSFV C-strain genetic make-up. This despite the fact that Flc6 and Flc9 needed adaptation to SK6.T7 cells and grow to lower titres than Flc7, Flc11 and Flc2.

Example 2 (animal experiment: 298-47042-00.22/97-11)
Pigs vaccinated with Flc9 and Flc11 are protected against a lethal challenge with virulent CSFV strain Brescia, and can be discriminated from infected animals by CSFV-specific E2 or E^{RNS} serology.

Materials and Methods

Animals

Specified-pathogen-free (SPF) pigs of 6-7 weeks of age were obtained from the SPF herd of the ID-DLO. Pigs were randomly divided in groups, and housed in separate stables of the high containment facilities of ID-DLO. The animals were left to acclimatise for four days before the first treatment. The

animals were fed once a day, in a trough, with complete food pellets, and could drink water from a nipple ad libitum.

Vaccination and challenge

On arrival, 27 SPF pigs were randomly divided in three groups: two groups of 10 pigs (A, B), and one group of 7 pigs (C). The pigs in group A were vaccinated with strain Flc 9; the pigs in group B were vaccinated with strain Flc 11. The vaccines were dissolved in an oil-in-water emulsion (DOE) (Hulst *et al.*, 1993. J.Virol. **67**:5435-5442). Each animal received 2 ml of the vaccine intramuscularly in the neck behind the ear. The pigs in group C remained unvaccinated.

Group A: Flc 9 (10)
Group B: Flc 11 (10)
Group C: controls (7)

Four weeks after vaccination, five animals in the groups A and B, and three animals in groups C were separated from their group and placed in a different, separate part of their stable. These pigs were challenged intranasally with 100 50% lethal doses (= 100 LD_{50}) of CSFV strain Brescia 456610 (Terpstra and Wensvoort, 1988. Vet. Microbiol. **16**:123-128). The pigs returned to their original group 24 h later. The pigs remained together during seven weeks.
Viral contents of the challenge inoculum were determined by titration of a sample taken after return from the stable.

Clinical observations

The pigs were checked daily by the animal technicians, abnormal findings were recorded and if necessary the supervising veterinarian was called. Each group was observed at least 15 minutes per day before and during feeding and cleansing of the stable.
A reduction in food uptake of the group or an individual animal was noted. Body-temperatures were recorded during

several days before and up to 20 days after challenge.

Blood analysis after challenge

EDTA-blood samples were collected on days 0, 2, 4, 7, 9, and 11 after challenge to monitor changes of leucocyte and trombocyte numbers in the blood. A decrease in the number of leucocytes (leucopenia) and thrombocytes (thrombocytopenia) is one of the typical signs of CSF. Normal cell counts for white blood cells and thrombocytes in conventional swine range between 11-23 10^9/l and 320-720 10^9 /l, respectively. For SPF pigs these values are a bit lower: 5-12 10^9/l and 200-700 10^9 /l, respectively. Both ranges mentioned vary in each pig. The bloodcell analyses were performed with a Medonic® CA 570 coulter counter. Leucopenia and thrombocytopenia were defined as cell/platelets counts considerably lower than the minimum number mentioned above, preferably for more than one day.

Virus isolation and viral antigen detection

Virus isolation: Peripheral blood leukocytes were extracted from EDTA-blood samples taken on day 0, 2, 4, 7, 9, and 11 after challenge to monitor viraemia. The samples were stored at -70 °C. The presence of CSFV in the leucocytes was examined as follows. In a M24 plate (Greiner), 300 µl (containing approximately 5×10^6 cells) of a swine kidney cell (SK6) suspension was added to each well and cultured at 37 °C and 5 % CO_2 in a humid chamber for 24 h. After 24 h, the medium was removed and 300 µl of an undiluted freeze/thawed leucocyte sample was added per well. After one hour of incubation at 37° C and 5% CO2, the sample was removed. The monolayer was then washed by adding and removing 400 µl of culture medium (Eagle basal medium). Subsequently, 800 µl of culture medium (Eagle basal medium with 5 % fetal bovine serum (FBS), free of pestivirus antibodies, and 1 % of an

antibiotic stock (containing ; glutamine (0.3 mg ml^{-1}), penicillin (200 units ml^{-1}), streptomycin (0.2 mg ml^{-1}) and mycostatin (100 U ml^{-1}.)) was added per well. After four days, the monolayers were washed in 10% NaCl solution, dried for 1 h at 80° C, incubated with a buffered solution containing CSFV specific conjugated antibodies, washed and stained. The monolayers were read microscopically for stained cells. Results were expressed as positive or negative for virus.

IFT: At post-mortem, tissue samples were collected from tonsil, spleen, kidney, and ileum, and were tested by direct immunofluorescent technique (Ressang and De Boer, 1967. Tijdschrift voor Diergeneeskunde **92**:567-586) for the presence of viral antigen. Cryostat sections (4 μm thick, two per organ) from these tissue samples were fixed and incubated with a polyclonal swine anti-pestivirus FITC-conjugated serum. After washing, the sections were read under a fluorescence microscope. Results were expressed as positive (= fluorescence) or negative (= no fluorescence).

Serological response

Serum blood samples of all pigs except the controls were collected at 0, 2, and 3 weeks after vaccination and at death (5 weeks after challenge). Samples were stored at -20 °C and assayed in a CSFV specific (Terpstra and Wensvoort. 1984. Vet. Microbiol. **33**:113-120) and 5250 specific (de Smit, unpublished) virus neutralization test (NPLA), in the Ceditest® ELISA for detecting CSFV specific antibodies against E2 (Colijn *et al.*, 1997. Vet. Microbiol. **59**:15-25), and in a Ceditest® ELISA for the detection of antibodies against E^{RNS} (de Smit *et al.*, in prep).

CSFV specific neutralizing antibody titres in serum were determined in a microtitre system. Serial twofold dilutions of serum were mixed with an equal volume of a CSFV (strain

Brescia) suspension which contained 30-300 $TCID_{50}$. After incubation for 1 hour at 37 °C in a CO_2 - incubator approximately 25,000 PK-15 cells per well were added. After four days, the microtitre plates were treated as mentioned above and read microscopically. The CSFV neutralizing titre was expressed as the reciprocal of the highest dilution that neutralized all virus.
Neutralizing antibody titres against 5250 in serum were determined similarly using the BVDV strain 5250 as antigen.
The CSFV E2-ELISA was performed according to the instructions of the manufacturer (Colijn *et al.*, 1997. ibid). The CSFV E^{RNS}-ELISA was performed as follows (de Smit *et al*, in prep). Test sera (30 µl) are pre-incubated with CSFV E^{RNS}-antigen (70 µl of a working dilution of baculovirus expressed E^{RNS} of CSFV strain Brescia) in a 96-wells non-coated microtitre plate containing 45 µl of ELISA buffer for 30 min at 37°C. Thereafter 50 µl of this pre-incubation mix is added to a microtitre plate coated with the E^{RNS}-specific monoclonal antibody 137.5, and containing 50 µl of a working solution of the peroxidase® conjugated E^{RNS}-specific monoclonal antibody 140.1.1. The plates are incubated for 1 h at 37°C, washed six times with 200 µl of washing solution, and incubated for 30 min at room temperature with 100 µl of a ready-to-use chromogen(3,3',5,5'-tetramethylbenzidine)/substrate solution. The colour reaction is stopped by adding 100 µl of a 0.5 M H_2SO_4 solution, and the optical density was measured at 450 nm using an Easy Reader® spectrophotometer (SLT Vienna).

Transmission of virus

The reproduction ratio *R* was used as measure for transmission. *R* is the number of secondary infections caused by one infectious animal. The seroconversion, body temperature, leucocyte- and thrombocyte counts, viremia, and antigen detection in tissues of the contact pigs were used to detect virus transmission by the vaccinated pigs after

challenge. *R* was estimated by determining the number of contact infections and subsequent analysis using a stochastic mathematical model (De Jong and Kimman, 1994. Vaccine **12**: 712-766). *R* is an indication of whether the infection will spread or will fade out. When $R < 1$, the infection will disappear from the population; when $R > 1$, the infection may spread. *R* is estimated as follows:

$$R = \frac{N}{T} \sum_{i=S_T+1}^{S_0} \frac{1}{i}$$

In this formula, S_0 is the initial number of contact pigs; N is the total number of pigs; T is the number of pigs that have been infectious in the course of the experiment ($T=x+I_0$); S_T is the number of pigs that are still susceptible at the end of the experiment.

Results

Clinical observation, viral antigen detection, leukocyte/trombocyte counts

After vaccination none of the animals developed clinical signs or fever. After challenge, all control pigs (group C) developed fever, and other clinical signs of CSF like anorexia, paralysis, thrombocytopenia and leucopenia (Table 2). The three inoculated pigs (nos. 621, 622, 625) developed fever two days after challenge. The four contact pigs developed fever after 7 days (624) or 8 days (623, 626, 627) after contact with the inoculated pigs. All pigs were killed in a moribund state 13 days after challenge. In group A (Flc9), all inoculated pigs (nos. 602, 606, 608, 609, 610) developed a mild fever (40 °C < T <41 °C) for one or more days, starting 3 days after challenge. In this group three contact-exposed pigs developed a mild fever, no. 604 at six days, no. 605 at seven days, and no. 607 at three days

after contact. Two contact pigs did not develop fever. In group B (Flc11), only one inoculated pig (605) had a very mild fever (40 °C) for one day. None of the other inoculated pigs nor the contact-exposed pigs developed fever.

In the control group (group C), all pigs developed trombocytopenia (< 180 cells/l); only three pigs (nos. 622, 623, 627) developed leucopenia ($<4.8\times10^9$ cells/l) , but in the other 4 pigs the number of leucocytes decreased (Table 2). None of the vaccinated pigs, either in group A (Flc9) or B (Flc11), developed leucopenia or trombocytopenia.

In the control group (group C), virus was detected in the leucocytes of all pigs, either inoculated or contact-exposed. Moreover, after killing of the pigs, all tissues of all pigs were IFT-positive (Table 2).
In both groups A and B (Flc9 and Flc11, respectively), no virus was detected in the leucocytes. Moreover, the tissues of all pigs were IFT-negative at the end of the experiment.

Serological response

All pigs in the control group (group C) died of CSF before developing any serological response.

After vaccination of the pigs in group A (Flc9), no CSFV-specific antibodies were detected (Table 3) with the E2-ELISA, whereas all pigs showed a low background inhibition percentage in the E^{RNS} ELISA (Table 4). This finding was consistent with the NPLA results: all vaccinated pigs remained negative for neutralising antibodies against CSFV (Table 5), and some (602, 603, 604, 607 and 608) developed low levels of neutralising antibodies against BVDV strain 5250 (Table 6). The latter was as expected since Flc9 contains the E2 gene, encoding the major antigenic protein of pestiviruses, of strain 5250.

After challenge inoculation, a high inhibition percentage in the E2-ELISA was observed in all inoculated pigs, and in two contact-exposed pigs (nos. 601, 603) (Table 3). Four of the inoculated pigs (nos. 606, 608, 609, 610) and one contact pig (no. 601) showed a high inhibition percentage in the E^{RNS} ELISA (Table 4). The fifth inoculated pig showed a lower, but positive inhibition percentage in the E^{RNS} ELISA, and the four remaining contact pigs showed intermediate inhibition percentages, considered negative.
In the neutralising antibody assays, all inoculated pigs and one contact pig (no. 601) showed high titres against CSFV. Two contact pigs (603, 607) showed an intermediate titre, and two remaining contact pigs did not develop antibodies against CSFV (Table 5). In the NPLA against 5250, four of the inoculated pigs (nos. 606, 608, 609, 610) developed high titres against 5250 after inoculation (Table 6). The titre in one inoculated pig remained low (no. 602). All contact pigs showed an increase in titre which is assumed to be due to continuation of the immune response against the vaccine virus Flc9.

After vaccination of the pigs in group B (Flc11), low percentages of CSFV-specific antibodies were detected with the E2-ELISA in some pigs (nos. 611, 615, 616-619). The other pigs remained negative (Table 3). In the E^{RNS} ELISA, all pigs showed a low to intermediate inhibition percentage after vaccination (Table 4). The NPLA results were as follows: some vaccinated pigs (nos. 611, 614, 615-619) developed low titres of neutralising antibodies against CSFV (Table 5), but some pigs (nos. 611-613, 616-617) also developed low titres against BVDV strain 5250 (Table 6).
After challenge inoculation, a high inhibition percentage in the E2-ELISA was observed in all pigs (Table 3). Four of the inoculated pigs (nos. 611, 615, 617, 618) showed a positive inhibition percentage in the E^{RNS}-ELISA (Table 4). The fifth inoculated pig (620) showed a lower, intermediate inhibition

percentage in the E^{RNS}-ELISA on the threshold of being positive. All contact pigs showed intermediate inhibition percentages in the E^{RNS}-ELISA at levels considered negative. In the neutralising antibody assays, all inoculated pigs developed high titres against CSFV (Table 5). None of the contact pigs showed high titres of neutralising antibodies against CSFV. Since neutralising antibodies are mainly directed against E2 of pestiviruses and strain 5250 is of a pestivirus type hardly cross-reacting serologically with CSFV, the results of the 5250 NPLA are low or negative for all animals in the Flc11 group.

Transmission of virus

Based on all variables determined in the contact pigs (mentioned in the Material and Method section), all four contact pigs in the control group (C) became infected, and two contact exposed pigs in the Flc 9 group (A) became infected. None of the contact pigs in the Flc11 group (B) became infected. Based on these results, the following reproduction rates were calculated:

$R_{control}$ 10

R_{flc9} 0.6

R_{flc11} 0

Description of the figures

Figure 1. Comparison of the deduced amino acid sequence of E^{RNS} (A) and E2 (B) proteins of the indicated pestiviruses. The relevant genomic regions of BVDV strain 5250 were sequenced and then compared to those of the type I BVDV strains NADL and Osloss, Border disease virus strain BD31, type II BVDV strain 890 and CSFV strains Brescia and C. The positions of amino acid residues are based on those of CSFV strain C (Moormann *et al.*, 1996. J. Virol.**70**:763-770; EMBL database nr. Z46258).

Figure 2. Schematic representation of the construction of pPRKflc6, pPRKflc7, pPRKflc9 and pPRKflc11. From the RNA genomes of BVDV strains 5250 and NADL fragments were amplified covering the E2 or E^{RNS} genes through RT/PCR (indicated by arrows). These fragments were cloned in pGEM4z-bleu (not shown) and through PCR cloned in transient expression vectors resulting in pPRKh1, pPRKc13 and pPRKh18. Expression of BVDV strain 5250 E^{RNS} and CSFV C-strain E2 genes in pPRKc13, and the strain 5250 E2 and C-strain E^{RNS} genes in pPRKh18, was studied by transient expression in SK6 cells. Expression of BVDV strain NADL E2 and C-strain E^{RNS} genes in pPRKh1, was studied by transient expression in SK6.T7 cells. Cloning of the *NcoI/XbaI* fragment from pPRc132 into pPRKh1 resulted in full-length clone pPRKflc6 (A). Cloning of the *ClaI/NgoMI* fragment from plasmid pPRKc13 into pPRKflc3 resulted in full-length clone pPRKflc7 (B). Cloning of the *ClaI/BglII* fragment of pPRKh18 into pPRKflc3 resulted in full-length clone pPRKflc9 (C). Cloning of the *ClaI/NgoMI* fragment of of pPRKc13 into pPRKflc2 resulted in full-length clone pPRKflc11 (D). N^{PRO}, autoprotease; C, core protein; E^{RNS}, E1 and E2, envelope proteins; NS2, NS23, NS3, NS4a, NS4b, NS5a, NS5b, non-structural genes; 5' NTR, 5' non-translated region; 3' NTR, 3' non-translated region; phCMV is the promotor-enhancer region of the immediate-early gene of human cytomegalovirus (hCMV); T7, bacteriophage T7 promotor sequence.

Figure 3. Multistep growthcurve of viruses Flc2, Flc9, Flc11 en 5250. Confluent monolayers of SK6 cells were infected with a multiplicity of infection of 0.1 with strains Flc2, Flc9, Flc11 and 5250. Virus titres were determined separately from supernatants and cell lysates. Virus titres (log TCID50 per millilitre) were established at 0, 6, 24, 48, 72, 96, 120 and 144 hours after infection.

Table 1. Characterization of chimeric CSFV/BVDV recombinant C-strain viruses

Reaction of CSFV and BVDV specific Mabs

strain	Directed against E2			Directed against E^{RNS}		
	BVDV specific epitope[a]	BVDV specific epitope[b]	C-strain specific epitope[c]	CSFV specific epitope[d]	BVDV specific epitope[e]	C-strain specific epitope[f]
FLc2	-	-	+	-	-	+
FLc6	+	+	-	-	-	+
FLc7	-	-	-	+	+	-
FLc9	-	+	-	-	-	+
FLc11	-	-	+	-	+	-
NADL	+	+	-	-	-	-
5250	-	+	-	-	+	-
Brescia	-	-	-	+	-	-

a) Mab CT6 specifically recognizes an epitope on E2 of BVDV (Bolin *et al.*, Arch. Virol. 99, 117-123, 1988).

b) Mab Wb166 specifically recognizes an epitope on E2 of BVDV different from Mab CT6 (Paton *et al.*, Virology 190, 763-772, 1992).

c) Mab C2 specifically recognizes an epitope on E2 of CSFV strain C (Wensvoort, G., Ph. D thesis. State University of Utrecht, Utrecht, The Netherlands 1989).

d) Mab b6 recognizes an epitope in domain B of E2 of CSFV strain Brescia (Wensvoort *et al.*, J. Gen. Virol. 70, 2865-2876, 1989).

e) Mab Wb433 specifically recognizes an epitope on E^{RNS} of BVDV (Paton *et al.*, Arch. Virol. 135, 241-252, 1994).

f) Mab C5 specifically recognizes an epitope on E^{RNS} of CSFV strain C. (Wensvoort, G., Ph.D. thesis. State University of Utrecht, Utrecht, The Netherlands, 1989).

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Table 2: Results of virus isolation, cytopenia and fever after challenge with CSFV

group	pig no.	I/C	# days with fever[a]	viremia	IFT	cytopenia[b]	death
A.	601	c	0	-	-	-	-
Flc9	602	i	1	-	-	-	-
	603	c	0	-	-	-	-
	604	c	4	-	-	-	-
	605	c	1	-	-	-	-
	606	i	6	-	-	-	-
	607	c	4	-	-	-	-
	608	i	3	-	-	-	-
	609	i	6	-	-	-	-
	610	i	6	-	-	-	-
B.	611	i	0	-	-	-	-
Flc11	612	c	0	-	-	-	-
	613	c	0	-	-	-	-
	614	c	0	-	-	-	-
	615	i	0	-	-	-	-
	616	c	0	-	-	-	-
	617	i	0	-	-	-	-
	618	i	0	-	-	-	-
	619	c	0	-	-	-	-
	620	i	0	-	-	-	-
C.	621	i	12	+	+	+	+
contr	622	i	12	+	+	+	+
	623	c	6	+	+	+	+
	624	c	7	+	+	+	+
	625	i	12	+	+	+	+
	626	c	6	+	+	+	+
	627	c	6	+	+	+	+

a) Fever: body temperature ≥40 °C

b) Cytopenia: thrombocytopenia and/or leucopenia

i: vaccinated and inoculated animal

c: vaccinated in contact animal

- : not detected/observed

+ : detected/observed

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Table 3: Results of the Ceditest® ELISA for the detection of CSFV-E2 antibodies[a]

Group	Animal no.	I/C	lab	days post challenge									
				-26	0	14	15	16	21	28	35	42	49
A.	601	c	1	0	0	0		19	78	90	94	99	100
Flc9	602	i	2	0	0	71			100	102	102	102	102
	603	c	3	0	0	0		0	6	53	90	87	93
	604	c	4	0	0	0		0	0	0	0	0	0
	605	c	5	0	0	0		0	0	0	0	0	0
	606	i	6	0	0	32			100	102	102	102	102
	607	c	7	0	0	0		0	0	0	0	0	0
	608	i	8	0	0	72			101	102	102	102	102
	609	i	9	0	0	58			100	102	102	102	102
	610	i	10	0	0	95			102	102	102	102	102
B.	611	i	11	0	40	102			101	101	102	102	102
Flc11	612	c	12	0	0	40		62	84	81	82	89	93
	613	c	13	0	8	63		34	79	85	78	85	88
	614	c	14	0	0	28		56	80	83	78	90	95
	615	i	15	0	11	102			102	102	102	102	102
	616	c	16	0	20	34		31	62	76	72	63	75
	617	i	17	0	35	103			102	102	102	102	102
	618	i	18	0	12	103			102	102	102	102	102
	619	c	19	0	14	45		52	86	77	79	75	82
	620	i	20	0	0	103			102	102	102	102	102
C.	621	i	21	0	0	0	0						
contr	622	i	22	0	0	0	0						
	623	c	23	0	0	0	0						
	624	c	24		0	0	0						
	625	i	25		0	0	0						
	626	c	26		0	0	0						
	627	c	27		0	0	0						

a) The Ceditest® E2-ELISA specifically detects antibodies against envelope protein E2 of CSFV. Test results are expressed as the percentage inhibition of a standard signal; <30% is negative , 30-50% inhibition is doubtful, >50% inhibition is positive.

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Table 4: Results of the Ceditest® ELISA for the detection of CSFV-E^{RNS} antibodies[a]

Group	Animal no.	I/C	lab	days post challenge									
				-26	0	14	15	16	21	28	35	42	49
A.	601	c	1	29	46	97		99	98	99	100	101	100
Flc9	602	i	2	24	38	0			8	17	53	58	62
	603	c	3	26	30	47		55	51	47	52	46	48
	604	c	4	20	36	7		39	33	33	33	45	42
	605	c	5	21	46	27		35	44	57	61	66	68
	606	i	6	28	44	97			99	101	101	101	101
	607	c	7	20	41	64		69	54	45	60	57	58
	608	i	8	21	30	93			93	93	94	95	94
	609	i	9	29	41	97			98	96	98	99	99
	610	i	10	20	15	102			102	102	102	.102	102
B.	611	i	11	23	36	67			41	56	68	73	74
Flc11	612	c	12	43	46	30		57	29	0	16	10	44
	613	c	13	28	13	21		45	0	0	0	0	19
	614	c	14	33	22	5		51	9	1	26	18	21
	615	i	15	35	29	2			61	72	80	79	77
	616	c	16	21	28	0		23	11	17	17	31	17
	617	i	17	50	17	6			21	43	48	54	64
	618	i	18	38	0	0			15	43	67	65	70
	619	c	19	18	0	49		7	0	3	18	0	29
	620	i	20	19	0	0			0	35	58	39	49
C.	621	i	21	21	30	52	45						
contr	622	i	22	14	28	45	40						
	623	c	23	46	31	32	47						
	624	c	24		27	33	50						
	625	i	25		38	31	53						
	626	c	26		34	22	41						
	627	c	27		27	21	40						

a) The Ceditest® E^{RNS}-ELISA specifically detects antibodies against envelope protein E^{RNS} of CSFV. The test results are expressed as the percentage inhibition of a standard signal; <50% is negative, ≥50% is positive.

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Table 5: Results of the NPLA for the detection of CSFV Brescia-specific neutralizing antibodies

Group	Animal no.	I/C	lab	days post challenge									
				-26	0	14	15	16	21	28	35	42	49
A.	601	c	1	<12,5	<12,5	100		200	400	400	1600	>1600	>1600
Flc9	602	i	2	<12,5	<12,5	100			400	600	>1600	1600	>1600
	603	c	3	<12,5	<12,5	25		19	100	100	600	200	400
	604	c	4	<12,5	<12,5	<12,5		<12,5	50	25	75	12,5	<12,5
	605	c	5	<12,5	<12,5	<12,5		<12,5	37	<12,5	37	<12,5	12,5
	606	i	6	<12,5	<12,.5	150			600	1600	1600	>1600	>1600
	607	c	7	<12,5	<12,5	150		<12,5	25	<12,5	300	75	50
	608	i	8	<12,5	<12,5	400			>1600	>1600	>1600	>1600	>1600
	609	i	9	<12,5	<12,5	800			1600	>1600	>1600	>1600	>1600
	610	i	10	<12,5	<12,5	800			800	>1600	>1600	>1600	>1600
B.	611	i	11	12,5	37	1600			>1600	>1600	>1600	>1600	>1600
Flc11	612	c	12	<12.5	<12,5	150		150	150	200	600	300	400
	613	c	13	<12.5	<12,5	75		50	37	75	75	100	150
	614	c	14	<12.5	12,5	50		50	150	400	400	400	800
	615	i	15	<12.5	12,5	>1600			>1600	>1600	>1600	>1600	1600
	616	c	16	<12.5	19	75		150	50	100	150	50	100
	617	i	17	19	19	>1600			>1600	>1600	>1600	>1600	1600
	618	i	18	<12.5	12,5	>1600			>1600	>1600	>1600	>1600	>1600
	619	c	19	<12.5	12,5	75		100	200	300	400	150	150
	620	i	20	<12.5	<12,5	>1600			1200	>1600	>1600	1600	>1600
C.	621	i	21	<12,5	<12,5	<12,5	<12,5						
contr	622	i	22	<12,5	<12,5	<12,5	<12,5						
	623	c	23	<12,5	<12,5	<12,5	<12,5						
	624	c	24		mv	<12,5	200						
	625	i	25		mv	<12,5	<12,5						
	626	c	26		mv	<12,5	<12,5						
	627	c	27		12,5	<12,5	<12,5						

mv: missing value

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Table 6: Results of the NPLA for the detection of BVDV 5250-specific neutralizing antibodies

Group	Animal no.	I/C	lab	days post challenge									
				-26	0	14	15	16	21	28	35	42	49
A.	601	c	1	<12,5	<12,5	75		200	300	300	200	600	200
Flc9	602	i	2	<12,5	25	50			100	37	75	37	25
	603	c	3	<12,5	37	75		400	600	400	600	200	200
	604	c	4	<12,5	12,5	100		50	50	100	150	100	37
	605	c	5	<12,5	<12,5	50		25	50	75	200	100	100
	606	i	6	<12,5	<12,.5	100			200	400	300	200	300
	607	c	7	<12,5	37	100		100	200	100	75	75	100
	608	i	8	<12,5	19	800			600	800	300	200	200
	609	i	9	<12,5	<12,5	1600			>1600	800	600	1200	800
	610	i	10	<12,5	<12,5	800			800	1200	600	300	300
B.	611	i	11	19	12,5	100			50	100	75	25	37
Flc11	612	c	12	<12.5	37	37		75	75	50	25	37	75
	613	c	13	<12.5	12,5	37		50	19	19	37	19	<12,5
	614	c	14	<12.5	<12,5	<12,5		<12,50	12,5	25	<12,5	<12,5	<12,5
	615	i	15	<12,5	<12,5	12,5			75	50	25	<12,5	<12,5
	616	c	16	12,5	19	<12,5		12,5	<12,5	<12,5	12,5	<12,5	<12,5
	617	i	17	19	37	37			150	50	50	25	25
	618	i	18	mv	<12,5	150			400	100	37	50	25
	619	c	19	<12.5	<12,5	25		19	75	37	19	<12,5	37
	620	i	20	<12.5	<12,5	50			75	100	150	50	<12,5
C.	621	i	21	<12,5	<12,5	<12,5	<12,5						
contr	622	i	22	<12,5	12,5	<12,5	<12,5						
	623	c	23	<12,5	mv	<12,5	<12,5						
	624	c	24		mv	<12,5	<12,5						
	625	i	25		mv	<12,5	<12,5						
	626	c	26		mv	<12,5	<12,5						
	627	c	27		<12,5	<12,5	<12,5						

mv:missing value

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Table 7: Results of leukocyte counts (no. of cells * 10^9 / l)

							dpc					
Group	animal	labnr	i/c	0	2	4	7	9	11	14	16	18
A	701	1	c				15.4	16.4	14.5	12.9	13.0	12.0
1 w flc9	702	2	c				9.5	10.2	9.9	6.2	12.2	8.9
	703	3	c				9.4	10.5	10.3	8.7	10.8	14.7
	704	4	c				7.8	9.8	7.8	8.5	8.4	10.9
	705	5	c				20.4	19.3	14.3	11.4	11.1	14.2
	706	6	i	12.1	11.7	8.3	10.9	15.3	14.4	13.3	11.8	14.4
	707	7	i	14.3	14.4	12.9	10.2	16.9	14.5	15.9	12.2	17.2
	708	8	i	12.3	12.3	11.2	10.0	16.0	14.4	11.0	13.5	18.8
	709	9	i	8.1	8.3	8.3	5.5	9.3	8.6	7.9	6.4	14.3
	710	10	i	8.7	9.0	8.5	7.4	9.2	8.8	7.5	4.3	9.5
B	711	11	c				8.2	9.6	13.4	12.7	8.7	8.9
2 w flc9	712	12	c				10.7	x	10.6	11.7	11.6	24.5
	713	13	c				8.2	8.3	9.9	10.5	11.4	18.9
	714	14	c				6.6	8.7	11.5	9.2	18.3	12.8
	715	15	c				7.3	8.8	7.6	8.8	12.0	15.5
	716	16	i	12.0	10.3	7.6	12.8	13.0	15.2	16.1	11.9	16.3
	717	17	i	9.0	6.4	5.3	12.2	10.5	9.9	10.9	9.3	10.2
	718	18	i	11.8	11.7	7.0	14.6	17.0	16.2	19.3	14.4	14.1
	719	19	i	11.1	10.5	5.6	10.9	15.8	15.4	11.5	10.5	8.0
	720	20	i	9.4	10.8	6.5	11.4	12.8	9.0	11.8	11.0	12.8
C	736	36	i	14.6	7.3	8.7	5.5	14.7	x			
controls	737	37	i	9.4	5.0	4.9	5.0	5.7	x			
	738	38	i	15.8	9.8	9.6	6.6	10.8	x			
	739	39	i	11.2	10.3	5.3	4.2	4.6	x			
	740	40	i	12.1	7.0	8.3	7.4	10.1	x			

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Table 8: Results of tromboocyte counts (no. of cells * 10^9 / l)

							dpc					
Group	animal	labnr	i/c	0	2	4	7	9	11	14	16	18
A	701	1	c				1008	911	816	792	799	803
1 w flc9	702	2	c				545	552	452	481	528	573
	703	3	c				630	700	670	607	561	671
	704	4	c				400	434	475	411	317	418
	705	5	c				797	740	736	611	543	593
	706	6	i	611	645	601	586	715	627	782	766	808
	707	7	i	611	780	717	588	634	815	639	599	698
	708	8	i	474	655	721	649	735	782	592	633	571
	709	9	i	662	631	651	640	609	671	571	588	573
	710	10	i	426	431	422	425	444	455	382	382	486
B	711	11	c				616	643	643	754	629	688
2 w flc9	712	12	c				490	x	365	540	344	470
	713	13	c				481	640	530	652	582	687
	714	14	c				547	535	480	530	481	579
	715	15	c				487	448	534	459	567	729
	716	16	i	430	501	421	618	484	462	618	387	416
	717	17	i	530	412	598	722	965	820	808	568	669
	718	18	i	384	598	432	604	709	644	651	565	511
	719	19	i	550	665	497	820	758	788	941	722	770
	720	20	i	530	648	421	596	578	666	775	534	661
C	736	36	i	493	484	389	92	45	x			
controls	737	37	i	487	350	145	52	50	x			
	738	38	i	482	392	137	43	49	x			
	739	39	i	475	339	269	21	15	x			
	740	40	i	440	384	283	34	30	x			

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Table 9: Results of virus isolation in the leukocytes

							dpc					
Group	animal	labnr	i/c	0	2	4	7	9	11	14	16	18
A	701	1	c				-	-	-	-	-	-
1 w flc9	702	2	c				-	-	-	-	-	-
	703	3	c				-	-	-	-	-	-
	704	4	c				-	-	-	-	-	-
	705	5	c				-	-	-	-	-	-
	706	6	i	-	-	-	-	-	-	-	-	-
	707	7	i	-	-	-	-	-	-	-	-	-
	708	8	i	-	-	-	-	-	-	-	-	-
	709	9	i	-	-	-	-	-	-	-	-	-
	710	10	i	-	-	-	-	-	-	-	-	-
B	711	11	c				-	-	-	-	-	-
2 w flc9	712	12	c				-	-	-	-	-	-
	713	13	c				-	-	-	-	-	-
	714	14	c				-	-	-	-	-	-
	715	15	c				-	-	-	-	-	-
	716	16	i	-	-	-	-	-	-	-	-	-
	717	17	i	-	-	-	-	-	-	-	-	-
	718	18	i	-	-	-	-	-	-	-	-	-
	719	19	i	-	-	-	-	-	-	-	-	-
	720	20	i	x	-	-	-	-	-	-	-	-
C	736	36	i	-	-	-	+	+				
controls	737	37	i	-	-	+	+	+				
	738	38	i	-	-	+	+	+				
	739	39	i	-	-	+	+	+				
	740	40	i	-	-	+	+	+				

x = unsuitable for testing

Animal experiment 298-47042-00.28/9804 and 298-47042-00.33/9815

Table 10: Results of the Ceditest ELISA for the detection of CSFV-E2 antibodies (% inhibition)

								wpc				
Group	animal	labnr	i/c	-14	-7	0	1	2	3	4	5	6
A.	701	1	c	0	0	0	0	0	0	0	0	0
Flc9	702	2	c	0	0	0	0	0	69	99	99	102
1 w	703	3	c	0	0	0	0	0	0	0	0	0
	704	4	c	0	0	0	0	0	0	4	4	1
	705	5	c	0	0	0	0	0	0	0	0	0
	706	6	i	0	0	0	0	46	100	102	100	103
	707	7	i	0	0	0	0	77	101	102	100	102
	708	8	i	0	0	0	0	93	100	102	100	102
	709	9	i	0	0	0	0	82	100	103	100	103
	710	10	i	0	0	0	0	66	100	99	98	100
B.	711	11	c		0	0	0	0	0	0	0	0
Flc9	712	12	c		0	0	0	30	10	0	0	0
2 w	713	13	c		0	0	0	0	0	0	0	0
	714	14	c		0	0	0	0	0	0	0	0
	715	15	c		0	0	0	0	0	0	0	0
	716	16	i		0	0	0	97	101	101	101	102
	717	17	i		0	0	14	94	100	100	100	101
	718	18	i		0	0	42	94	100	101	101	101
	719	19	i		0	0	58	99	101	102	101	102
	720	20	i		0	0	0	98	102	103	100	101
								20-11				
C.	736	36	i			0	0	0				
Controls	737	37	i			0	0	0				
	738	38	i			0	0	0				
	739	39	i			0	0	0				
	740	40	i			0	0	0				

percentage inhibition < 50% is negative, ≥ 50% is positive

Animal experiment 298-47042-00.28/9804 and 298-47042-00.33/9815

Table 11: Results of the Ceditest ELISA for the detection of CSFV-E^{RNS} antibodies (% inhibition)

								wpc				
Group	animal	labnr	i/c	-14	-7	0	1	2	3	4	5	6
A.	701	1	c	13	14	50	52	31	35	37	38	69
Flc9	702	2	c	2	12	29	79	44	86	96	97	101
1 w	703	3	c	2	13	26	48	56	51	61	60	65
	704	4	c	3	0	17	51	55	54	63	76	84
	705	5	c	0	18	38	30	37	53	71	84	91
	706	6	i	0	3	28	62	92	92	97	97	98
	707	7	i	15	7	29	73	96	96	100	101	103
	708	8	i	15	19	37	25	81	88	97	95	100
	709	9	i	5	23	38	86	96	92	98	98	101
	710	10	i	12	2	13	90	91	84	90	92	92
B.	711	11	c		20	61	58	58	58	68	72	76
Flc9	712	12	c		14	44	51	56	47	56	56	59
2 w	713	13	c		8	54	42	60	53	75	75	78
	714	14	c		5	49	43	45	26	39	62	42
	715	15	c		8	39	29	48	45	61	71	74
	716	16	i		0	40	87	76	61	70	73	77
	717	17	i		18	43	79	71	88	96	98	100
	718	18	i		19	59	82	83	93	96	100	96
	719	19	i		9	49	97	94	90	91	95	97
	720	20	i		6	24	98	87	90	95	97	99
								20-11				
C.	736	36	i			14	32	7				
Controls	737	37	i			12	2	2				
	738	38	i			17	0	5				
	739	39	i			24	2	1				
	740	40	i			19	0	2				

percentage inhibition <50% is negative, ≥ 50% is positive

Animal experiment 298-47042-00.28/9804 and 298-47042-00.33/9815

Table 12: Results of the VP NPLA for the detection of CSFV Brescia-specific neutralising antibodies

								wpc				
Group	animal	labnr	i/c	-2	-1	0	1	2	3	4	5	6
A	701	1	c	<10	<10	<10	<10	10	<10	<10	10	10
1 w flc9	702	2	c	<10	<10	<10	15	30	960	1280	960	15360
	703	3	c	<10	<10	<10	10	<10	10	15	10	15
	704	4	c	<10	<10	<10	15	<10	10	10	<10	10
	705	5	c	<10	<10	<10	15	20	10	15	15	15
	706	6	i	<10	<10	<10	15	80	480	>1280	1280	5120
	707	7	i	<10	<10	<10	<10	120	640	>1280	1280	5120
	708	8	i	<10	<10	<10	<10	960	960	>1280	>1280	3840
	709	9	i	<10	<10	<10	>20	320	320	>1280	>1280	10240
	710	10	i	<10	<10	<10	15	80	240	1280	480	1920
B	711	11	c		<10	10	<10	<10	10	10	15	10
2 w flc9	712	12	c		<10	<10	<10	<10	<10	10	15	<10
	713	13	c		<10	<10	<10	10	10	15	15	10
	714	14	c		<10	<10	<10	<10	<10	<10	10	<10
	715	15	c		<10	<10	10	<10	10	<10	10	<10
	716	16	i		<10	<10	<10	>1280	1280	>1280	1280	5120
	717	17	i		<10	<10	>20	960	>1280	>1280	>1280	10240
	718	18	i		<10	15	>20	960	>1280	>1280	>1280	5120
	719	19	i		<10	<10	>20	>1280	1280	>1280	>1280	3840
	720	20	i		<10	<10	>20	320	>1280	>1280	>1280	7680
								20-11				
C	736	36	i			<10	<10	<10				
controls	737	37	i			<10	<10	<10				
	738	38	i			<10	<10	<10				
	739	39	i			<10	<10	<10				
	740	40	i			<10	<10	<10				

Animal experiment 298-47042-00.28/9804 and 298-47042-00.33/9815

Table 13: Results of the 5250 NPLA for the detection of 5250-specific neutralising antibodies

							wpc					
group	animal	labnr	i/c	-2	-1	0	1	2	3	4	5	6
A	701	1	c	< 10	< 10	< 10	60	60	60	320	160	320
1 wk flc9	702	2	c	< 10	< 10	< 10	20	30	120	480	240	480
	703	3	c	< 10	< 10	< 10	120	60	120	320	960	240
	704	4	c	< 10	< 10	< 10	10	20	60	120	240	80
	705	5	c	< 10	< 10	< 10	15	40	60	240	320	320
	706	6	i	< 10	< 10	< 10	< 10	< 10	60	80	120	120
	707	7	i	< 10	< 10	< 10	30	40	120	480	240	320
	708	8	i	< 10	< 10	< 10	15	< 10	160	160	640	320
	709	9	i	< 10	< 10	< 10	30	40	80	320	640	640
	710	10	i	< 10	< 10	< 10	20	15	40	40	80	80
B	711	11	c		< 10	10	30	40	120	160	320	120
2 wk flc9	712	12	c		< 10	< 10	10	20	120	80	160	120
	713	13	c		< 10	< 10	40	30	40	120	120	160
	714	14	c		< 10	30	30	20	20	60	120	60
	715	15	c		< 10	20	40	60	60	240	320	160
	716	16	i		< 10	< 10	< 10	15	20	120	240	60
	717	17	i		< 10	15	60	120	480	320	960	480
	718	18	i		< 10	30	120	60	160	240	480	320
	719	19	i		< 10	< 10	80	320	320	320	640	480
	720	20	i		< 10	< 10	< 10	40	60	120	80	80
C	736	36	i			< 10	< 10	< 10				
control	737	37	i			< 10	< 10	< 10				
	738	38	i			< 10	< 10	< 10				
	739	39	i			< 10	< 10	< 10				
	740	40	i			< 10	< 10	< 10				

CLAIMS

1. A recombinant nucleic acid derived from a genotype of a pestivirus wherein a first fragment or part thereof encoding at least one immunodominant part at least partly responsible for providing protection against a wild-type pestivirus infection of a viral protein has been modified to render said immunodominant part incapable of eliciting antibodies specific for said genotype.

2. A recombinant nucleic acid derived from a genotype of a pestivirus wherein a first fragment or part thereof encoding at least one immunodominant part at least partly responsible for providing protection against a wild-type pestivirus infection of a viral protein has been replaced by a second fragment or part thereof encoding an antigenically unrelated part of a protein from another genotype pestivirus.

3. A nucleic acid according to claim 1 or 2 wherein said viral protein is an E2 protein.

4. A nucleic acid according to claim 3 wherein said first fragment comprises a nucleic acid encoding E2 protein running from about amino acid position 690 to 865 or 692 to 877 or 690 to 1008.

5. A nucleic acid according to claim 2 wherein said protein from another genotype pestivirus is an E2 protein.

6. A nucleic acid according to claim 5 wherein said second fragment comprises a nucleic acid encoding E2 protein running from about amino acid position 690 to 865 or 692 to 877 or 690 to 1008.

7. A nucleic acid according to claim 1 or 2 wherein said viral protein is an E^{rns} protein.

8. A nucleic acid according to claim 7 wherein said first fragment comprises a nucleic acid fragment encoding E^{RNS} protein running from about amino acid position 268 to 494.

9. A nucleic acid according to claim 2 wherein said protein from another genotype pestivirus is an E^{rns} protein.

10. A nucleic acid according to claim 9 wherein said second fragment comprises a nucleic acid encoding E^{RNS} protein running from about amino acid position 268 to 494.

11. A chimeric pestivirus comprising a nucleic acid according to anyone of claims 1 to 10.

12. A chimeric pestivirus according to claim 11 wherein said chimeric pestivirus is deposited as I-2058 at 31 July 1998 at the CNCM, Paris, France.

13. A chimeric pestivirus according to claim 11 wherein said chimeric pestivirus is deposited as I-2059 at 31 July 1998 at the CNCM, Paris, France.

14. A chimeric pestivirus according to claim 11 wherein said chimeric pestivirus is deposited as I-2060 at 31 July 1998 at the CNCM, Paris, France.

15. A chimeric pestivirus according to claim 11 wherein said chimeric pestivirus is deposited as I-2061 at 31 July 1998 at the CNCM, Paris, France.

16. A vaccine comprising a chimeric pestivirus according to anyone of claims 11 to 15 allowing serological discrimination of an animal vaccinated with said vaccine from an animal infected with wild-type pestivirus.

17. A vaccine according to claim 16 capable of providing sufficient herd immunity to a vaccinated population to reduce the reproduction ratio (R_0) of said wild-type pestivirus to at least below 1.

18. A vaccine according to claim 16 or 17 wherein said chimeric pestivirus is derived from a pestivirus vaccine strain, such as C-strain virus.

19. A vaccine according to anyone of claims 16 to 18 wherein said wild-type pestivirus is a classical swine fever virus.

20. A vaccine according to anyone of claims 16 to 18 wherein said wild-type pestivirus is a bovine viral diarrhoea virus.

21. A vaccine according to anyone of claims 16 to 18 wherein said wild-type pestivirus is a Border disease virus.

22. A method for controlling and/or eradicating a wild-type pestivirus infection comprising vaccinating at least a part

of an animal population with a vaccine according to anyone of claims 16 to 21 and further comprising testing at least a part of said population for the presence of antibodies specific for said wild-type pestivirus.

Figure 1B

```
NADL            HLDCKPEFSYAIAKDERIGQLGAEGLTTTWKEYSPGMKLEDTMVIAWCEDGKLMYLQRCT
Osloss          LPV"""G"Y"""""NNE""P"""T""""Q"Y"""D""R"Q""G"VV""KG"EIK""IT"E
BD31            EFA"REDHR""L""TKE""P""""S"""""TD"QRSFD"D"GA"R"S"VG"YFRFREH"L
890             FPE""EG"Q"""S""RKM"L""P"S"""""HRPT__K""V"S""QV"""GKD"KI"KT"P
5250            FPE""EG"Q"""S""KK""L""P"S"""""HLPT__K""V"S""QV"""GKD"KI"KT""
Brescia         R"A""EDHR"""STTNE""LH""""""""""""NHNLQ"D"GT"K"I"MA"SFKVTALNV
C-strain    690 R"A""EDYR"""SSTDE""L"""G"""""""""NHDLQ"N"GT"K"S"VA"SFKVTALNV

NADL            RETRYLAILHTRALPTSVVFKKLFDGRKQEDVVEMNDNFEFGLCPCDAKPIVRGKFNTTL
Osloss          ""A"""""""""""""""""E"II""KE"""""""D""""L"""""""""L"""""""""
BD31            IGS""""S""Q"""""""T"ELIPG"STLVEE_""G"D"""""""""SR"L""""Y"A""
890             K"E"""VAV"E"""S""AE"MPIS""TIGP""ID"P"D""""""""""""VIK""""AS"
5250            ""E"""VAV"E"""S""AE"MQIS""KLGPS"ID"P"D"""""""""S""VIK""""AS"
Brescia         VSR""""S""KD""""""T"EL""""TSPLTE_""G"D"G"""""F"TS"V"K""F""""
C-strain        VSR""""S""KK""""""T"EL""""TNPSTE_""G"D"RS""""F"TS"V"K""Y""""

NADL            LNGPAFQMVCPIGWTGTVS_CTSFNMDTLATTVVRTYRRSKPFPHRQGCITQKNLGED
Osloss          """"""""""""""""""L"HWS"K""""M""""""K"HR"""F"""""""""VI"G"
BD31            """S"""L"""YE"""R"E_""TISKS""""E""KI"K"T"R"RSGLVATHTTIYE""
890             """""""""""Q"""""IE_""LA"Q"""D"""""""""TT""QR"KW"SYE"II"""
5250            """""""""""Q""""RIE_"""V"Q"""D"""""""""TT""QR"RW"VYE"MI"""
Brescia         """S""YL""""""""VIE_""AVSPT""R"E""K"F""E""""Y"RD"V"TTVEN""
C-strain        """S""YL""""""""VIE_""AVSPT""R"E""K"F""D""""""MD"V"TTVEN"" 865
```

Figure 1A

```
NADL              ENITQWNLQDNGTEGIQRAMFQRGVNRSLHGIWPEKICTGVPSHLATDIELKTIHGMMDA
Osloss            ################################################T"""A"""""""
BD31              """"""""S""""H"""H""""""""""""""""""""""""T"""""T""RG"Q"""""
890               """"""""M""""""""Q"""L""""""""""""""""""""T"""""Y""RE"V"""""
5250              """"""""M""""""""""""L""""""""""""""""""I"T"""""Y"""E"V"""""
C-strain     268  """"""""S""""N"""H""YL""""""""""""""""K"""T"""""V"""E"Q"""""

NADL              SEKTNYTCCRLQRHEWNKHGWCNWYNIEPWILVMNRTQANLTEGQPPRECAVTCRYDRAS
Osloss            """""""""""""""""""""""""""""""VL""K"""""A""""""""""""""""D"
BD31              """""""""""""""""""""""""""N"""W"""K""""""""P"EK""T""""F"KE"
890               """"""""""""""""""""""""FH"""""WL""K""N""""""""K"""""""""KEA
5250              """"""""""""""""""""""""FH"""""WL""K""S"""""""L"""""""""""ET
C-strain          ""G""""""K""""""""""""""H""D"""QL""""""D"A""P"VK"""""""""KDA

NADL              DLNVVTQARDSPTPLTGCKKGKNFSFAGILMRGPCNFEIAASDVLFKEHERISMFQDTTL
Osloss            """""""""N""""""""""""""""""V"VQ""""""""V"""""R""DCT"VI"G"AH
BD31              "M""""""""R""T""""""""K"""""MIIE"""""NVSVE"I""GDS"CS"L""""A"
890               E""I""""""R""T""""""""""""""VILD"""""KVSVE"""""""DCGN"L"E"AI
5250              E""I""""""R""T"""""""""""""""VLN"""""KVSVEE""""""DCGN"L"E"AI
C-strain          "I"""""""NR""T""""""""""""""TVIES""""NVSVE"T"YGD""CG"LL""AA"

NADL              YLVDGLTNSLEGARQGTAKLTTWLGKQLGILGKKLENKSKTWFGAYA
Osloss            """""M"""""S"""""""""""""R""KK"""""""""""""""""
BD31              "V"""V""TV"S""""A""""S""""""""M"""""H""""""""N"
890               Q"L""A""TI""""V"""""""""""""""""""""""T"A""""H"
5250              Q"L""A""TI""""V"""""""""""""R"""R"""""""S""""H"
C-strain          """""M""TI"N""""A"RV"S"""R""RTA"R"""GR"""""""""  494
```

Figure 2A

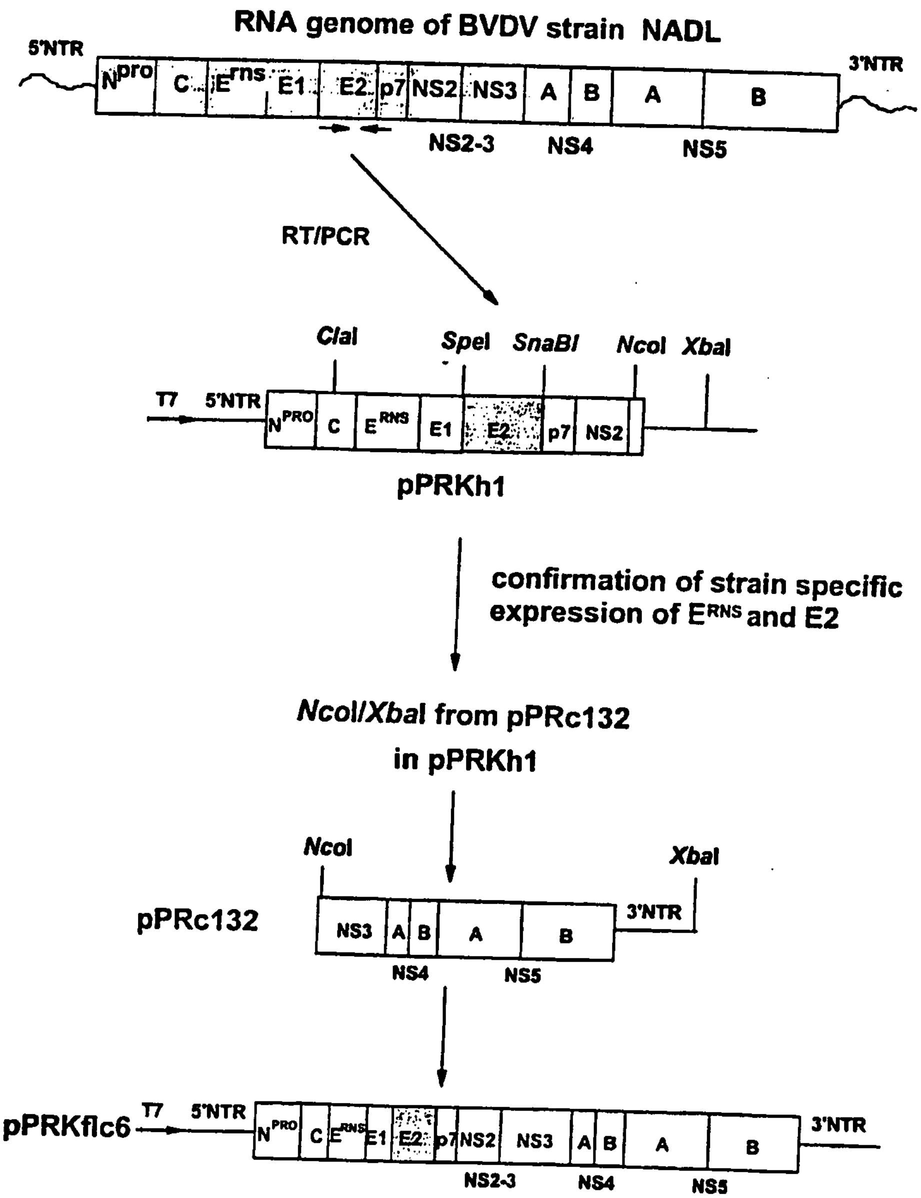

RNA genome of BVDV strain NADL
5'NTR
3'NTR
Npro
C
Erns
E1
E2
p7
NS2
NS3
A
B
A
B
NS2-3
NS4
NS5
RT/PCR
ClaI
SpeI
SnaBI
NcoI
XbaI
T7
5'NTR
NPRO
C
ERNS
E1
E2
p7
NS2
pPRKh1
confirmation of strain specific expression of ERNS and E2
NcoI/XbaI from pPRc132
in pPRKh1
NcoI
XbaI
pPRc132
NS3
A
B
A
B
3'NTR
NS4
NS5
pPRKflc6
T7
5'NTR
NPRO
C
ERNS
E1
E2
p7
NS2
NS3
A
B
A
B
3'NTR
NS2-3
NS4
NS5

Figure 2B

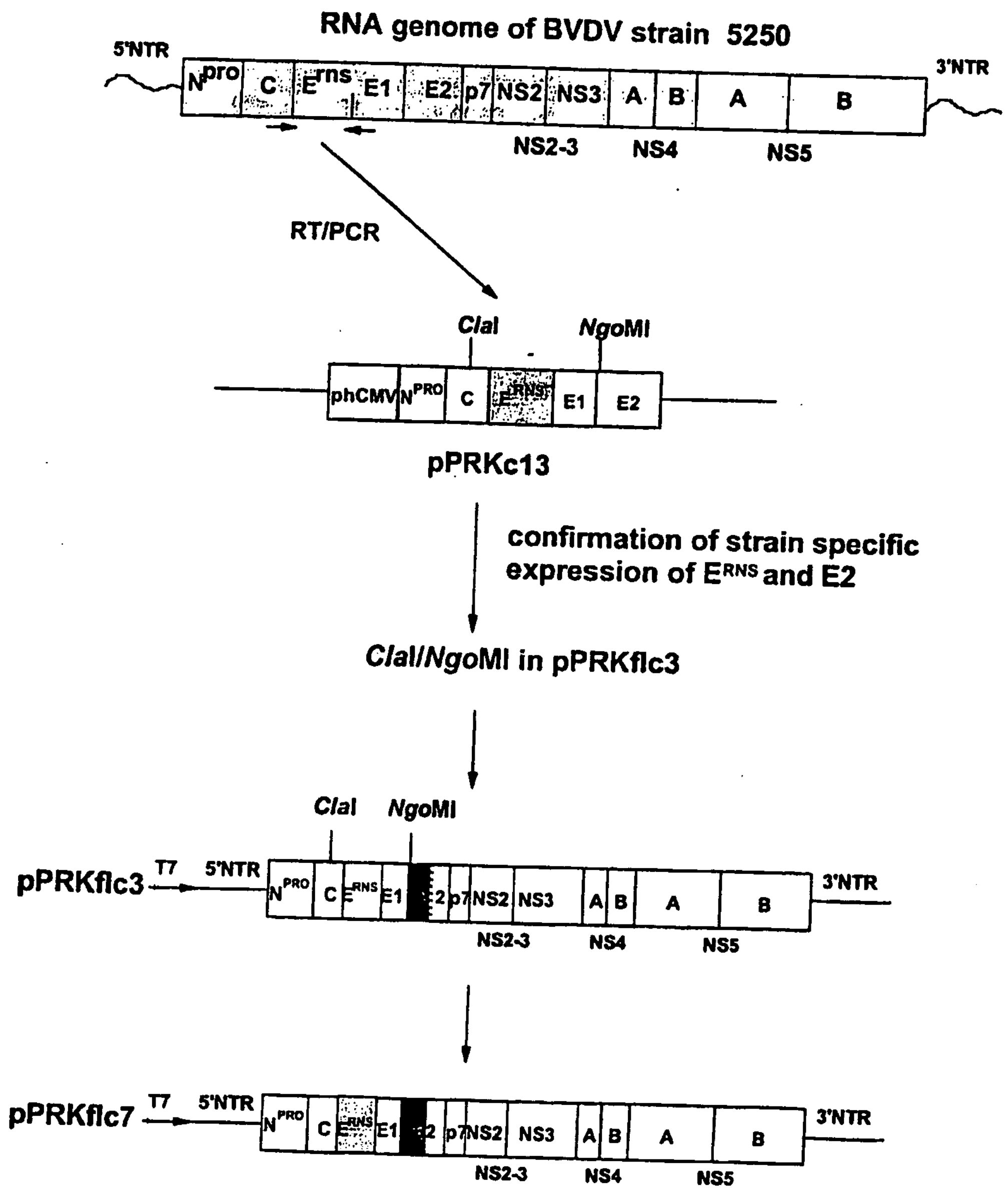

RNA genome of BVDV strain 5250
5'NTR
3'NTR
Npro
C
Erns
E1
E2
p7
NS2
NS3
A
B
A
B
NS2-3
NS4
NS5
RT/PCR
ClaI
NgoMI
phCMV
NPRO
C
ERNS
E1
E2
pPRKc13
confirmation of strain specific expression of ERNS and E2
ClaI/NgoMI in pPRKflc3
ClaI
NgoMI
pPRKflc3
T7
5'NTR
NPRO
C
ERNS
E1
E2
p7
NS2
NS3
A
B
A
B
3'NTR
NS2-3
NS4
NS5
pPRKflc7
T7
5'NTR
NPRO
C
ERNS
E1
E2
p7
NS2
NS3
A
B
A
B
3'NTR
NS2-3
NS4
NS5

Figure 2C

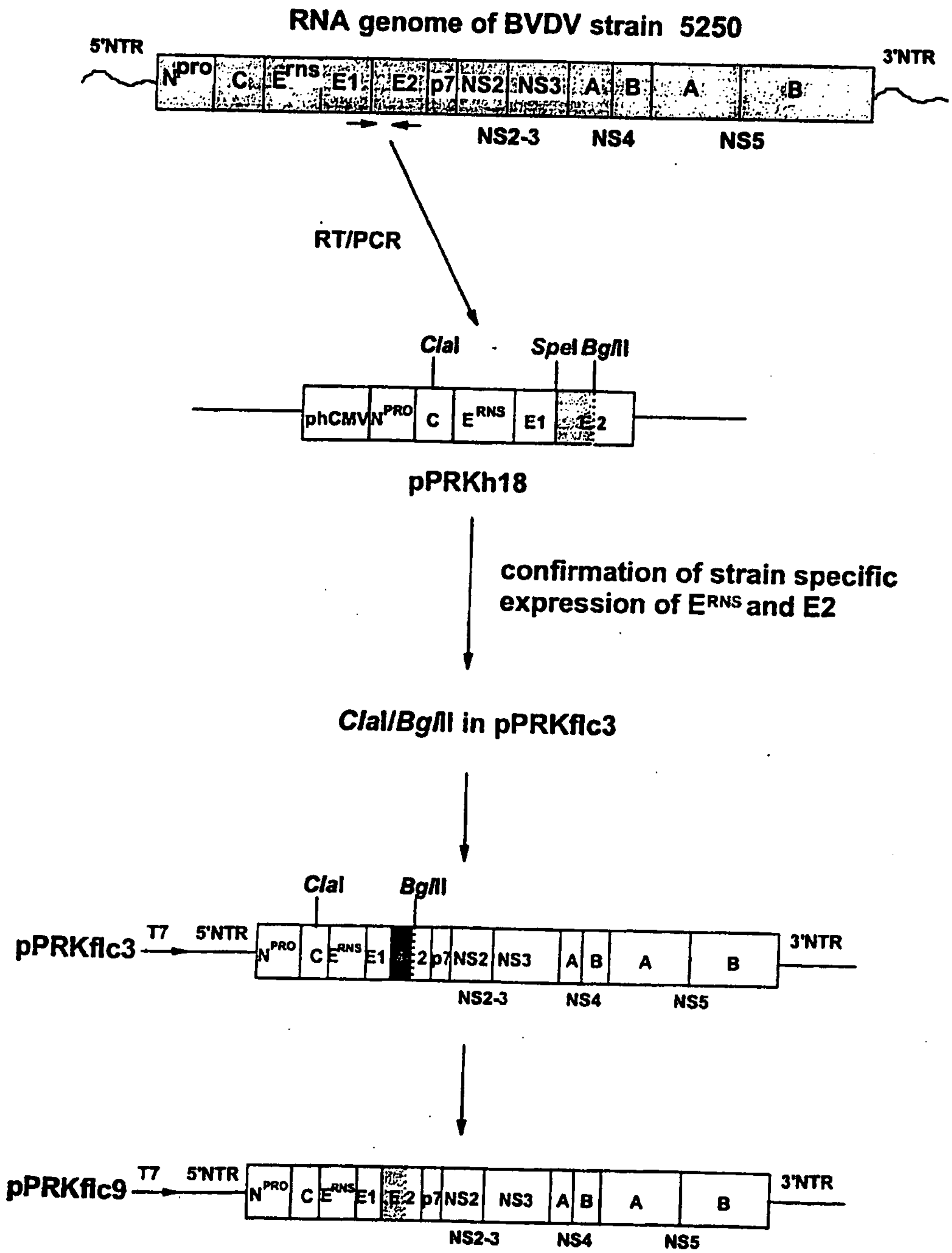
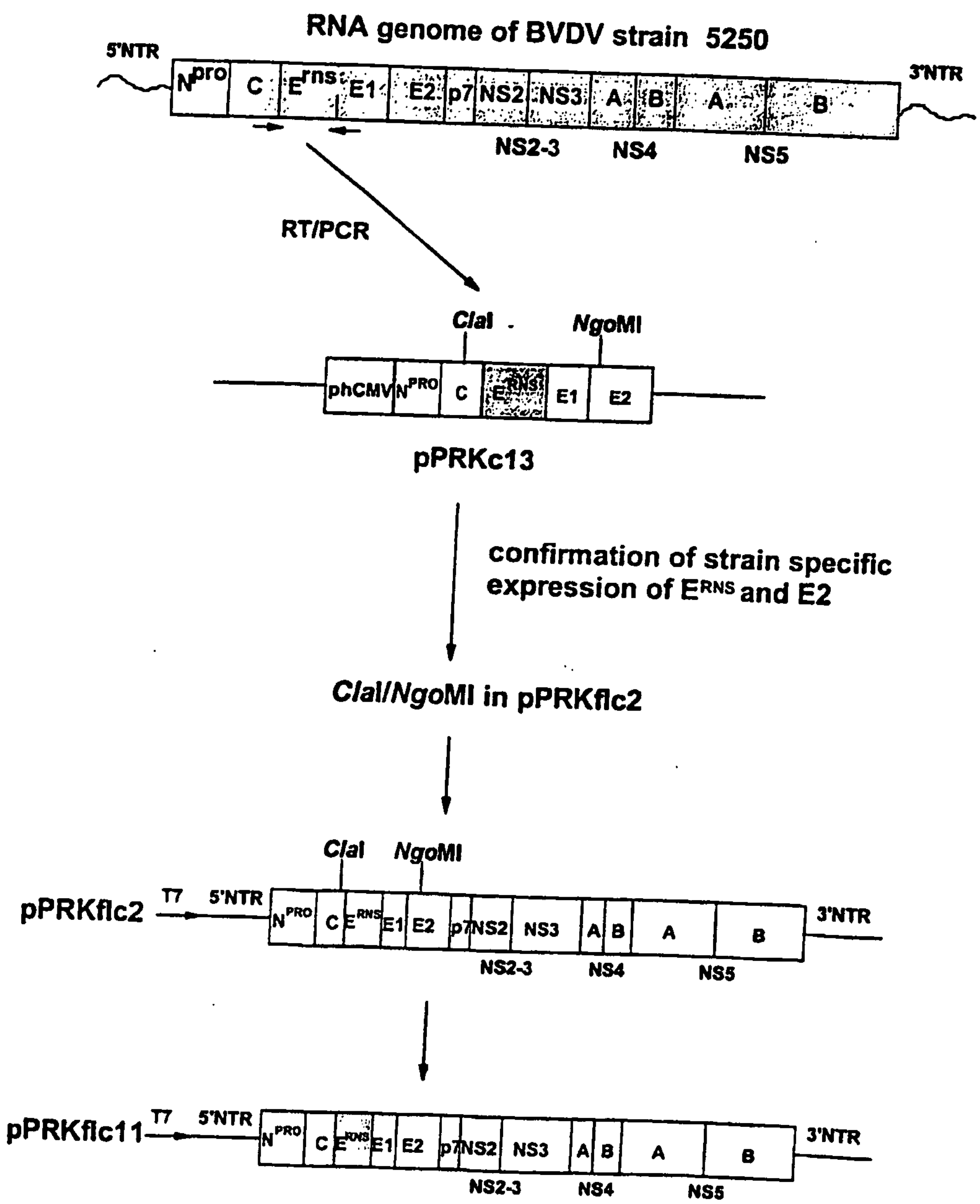

RNA genome of BVDV strain 5250
5'NTR
3'NTR
Npro
C
Erns
E1
E2
p7
NS2
NS3
A
B
A
B
NS2-3
NS4
NS5
RT/PCR
ClaI
SpeI
BglII
phCMV
NPRO
C
ERNS
E1
E2
pPRKh18
confirmation of strain specific expression of ERNS and E2
ClaI/BglII in pPRKflc3
ClaI
BglII
pPRKflc3
T7
5'NTR
NPRO
C
ERNS
E1
2
p7
NS2
NS3
A
B
A
B
3'NTR
NS2-3
NS4
NS5
pPRKflc9
T7
5'NTR
NPRO
C
ERNS
E1
E2
p7
NS2
NS3
A
B
A
B
3'NTR
NS2-3
NS4
NS5

Figure 2D


RNA genome of BVDV strain 5250
5'NTR
3'NTR
Npro
C
Erns
E1
E2
p7
NS2
NS3
A
B
A
B
NS2-3
NS4
NS5
RT/PCR
ClaI
NgoMI
phCMV
NPRO
C
ERNS
E1
E2
pPRKc13
confirmation of strain specific
expression of ERNS and E2
ClaI/NgoMI in pPRKflc2
ClaI
NgoMI
pPRKflc2
T7
5'NTR
NPRO
C
ERNS
E1
E2
p7
NS2
NS3
A
B
A
B
3'NTR
NS2-3
NS4
NS5
pPRKflc11
T7
5'NTR
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A
B
3'NTR
NS2-3
NS4
NS5

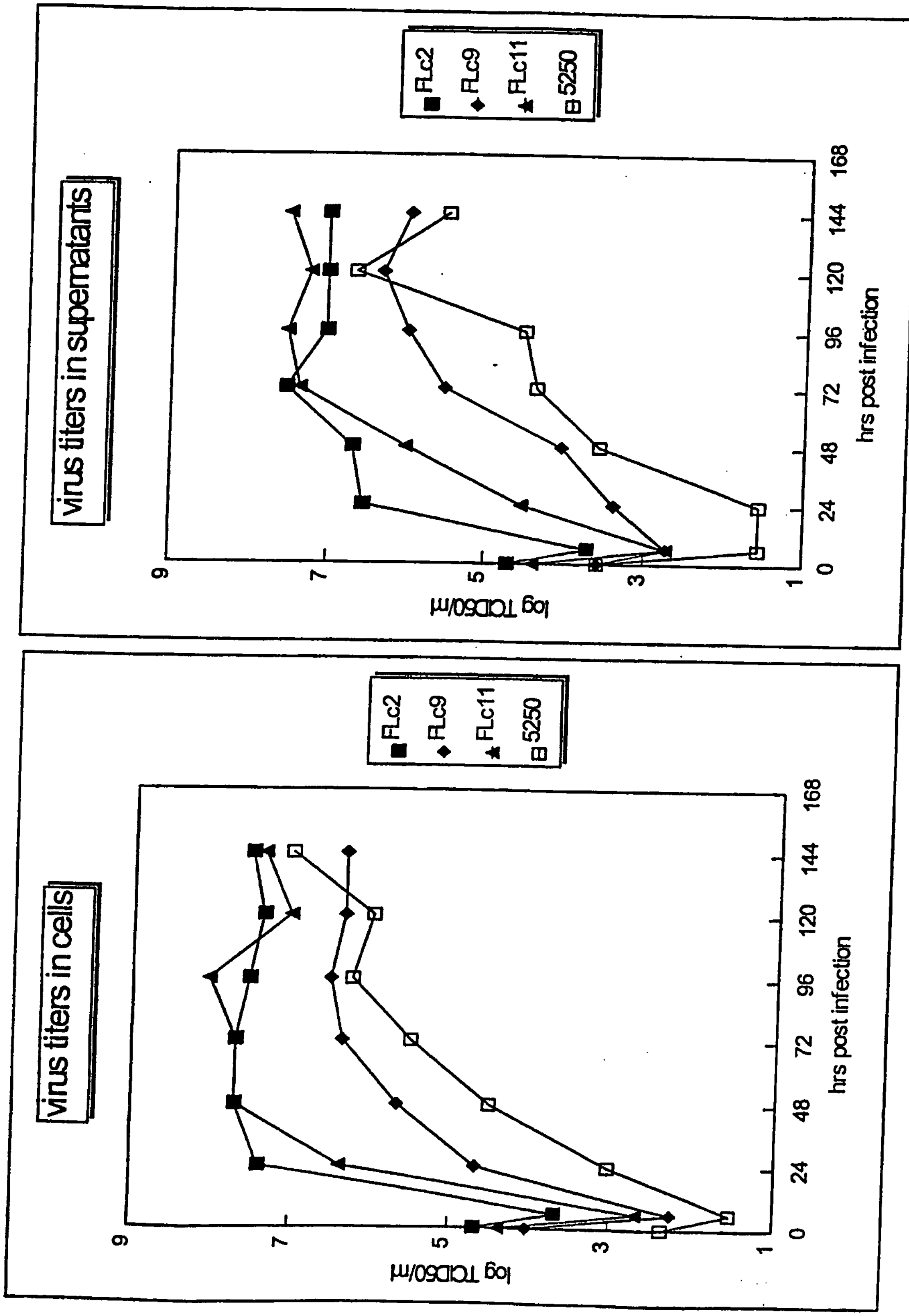
virus titers in supernatants
log TCID50/ml
hrs post infection
FLc2
FLc9
FLc11
5250
virus titers in cells
log TCID50/ml
hrs post infection
FLc2
FLc9
FLc11
5250
9
7
5
3
1
0
24
48
72
96
120
144
168

Figure 3

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/NL 99/00516

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/40 C07K14/08 C12N7/01 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	R.J.M. MOORMANN ET AL.: "Infectious RNA transcribed from am engineered full-length cDNA template of the genome of a Pestivirus" JOURNAL OF VIROLOGY., vol. 70, no. 2, February 1996 (1996-02), pages 763-770, XP002089686 cited in the application	1-3,5,7, 9,11-22
Y	page 769, right-hand column, paragraph 2 page 767, right-hand column, paragraph 3 -page 768, left-hand column, paragraph 5 page 763, right-hand column, paragraph 3 -page 764, left-hand column, paragraph 1 abstract	4,6

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 December 1999	21/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

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Inte ...onal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 95 35380 A (INSTITUUT VOOR VEEHOUDERIJ EN DIERGEZONDHEID) 28 December 1995 (1995-12-28) page 2, line 24 -page 3, line 23 page 3, line 29 -page 4, line 12 page 6, line 11 -page 8, line 27 page 9, line 30 -page 10, line 14 page 13, line 3 - line 11 page 13, line 26 -page 14, line 32; example 5 ---	1-3,5, 11-22 4,6
Y	P.A. VAN RIJN ET AL.: "Subdivision of the Pestivirus genus based on envelope glycoprotein E2" VIROLOGY, vol. 237, no. 2, 27 October 1997 (1997-10-27), pages 337-348, XP002089687 ORLANDO US cited in the application page 338, left-hand column, paragraph 2 page 341, left-hand column, paragraph 2 -page 344, left-hand column, paragraph 1 ---	4,6
A	R.J.M. MOORMANN ET AL.: "Recent developments in pig vaccination" PROCEEDINGS OF THE 14TH IPVS CONGRESS, 7 - 10 July 1996, pages 25-29, XP002089688 Bologna, Italy cited in the application the whole document -----	1-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL 99/00516

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 22 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 99/00516

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9535380 A	28-12-1995	AU 697866 B	22-10-1998
		AU 2631595 A	15-01-1996
		BR 9508054 A	12-08-1997
		CA 2192940 A	28-12-1995
		CN 1151185 A	04-06-1997
		CZ 9603696 A	11-06-1997
		EP 0766740 A	09-04-1997
		HU 76352 A	28-08-1997
		JP 9511914 T	02-12-1997
		NZ 287563 A	26-08-1998
		PL 317755 A	28-04-1997
		ZA 9505042 A	08-02-1996